AN INVESTIGATION OF THE RELATIONSHIP BETWEEN TUMOUR PROLIFERATION, SYSTEMIC AND LOCAL INFLAMMATORY RESPONSES AND SURVIVAL IN PATIENTS WITH TRANSITIONAL CELL CARCINOMA OF THE BLADDER

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DEDICATION

I dedicate this thesis to my mother and father for their encouragement. Also I would like to dedicate this thesis to my wife Rafal for her support and patience, without whom this work would not have been possible, and to my two daughters, Taiba and Shahad, and my son Yusef.

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DECLARATION

I declare that all the work presented in this thesis has been carried out solely by me except where indicated below. The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part, for any other degree or other qualification.

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SUMMARY

Bladder cancer is the fourth most common malignancy in the Western world. In the UK there are 12,500 new cases each year and 5,000 deaths annually. In 80% of patients the disease is diagnosed in its early superficial stage (Ta, T1) and 20% in its deep stage (invasive) T2-T4. Of the superficial group, 20% will never have recurrence, 50% will have one or more superficial recurrences within 1-2 years and 30% will develop invasive disease, most of whom will die from bladder cancer. This means that these patients need lifelong medical vigilance involving periodic internal inspection of their bladder. These constitute a significant proportion of the general urological consultants workload due to high prevalence and recurrent nature.

This has fuelled the continuous investigation of factors to predict those patients in whom who are likely to recur and those likely to progress. In identifying those patients most likely to recur and progress, one might significantly reduce mortality and also help to highlight those patients in whom intensive follow-up is not required.

It is recognised that both innate and adaptive immunity are involved in the immune response to bladder tumours. There is increasing evidence that cell-mediated immunity plays a key role in tumour progression of patients with bladder cancer. The infiltration of bladder tumours with tissue lymphocytes has been demonstrated and suggests the presence of a local active immune defence of the host against bladder tumour. In addition, some components of innate immunity have been found to be of prognostic value in bladder cancer. However, the relationship between the tumour proliferation, the systemic and local inflammatory responses and outcome remains unclear.

The thesis involved three cohorts of patients with transitional cell carcinoma of the urinary bladder. The first cohort consisted of patients in whom either pre- and/or

post resection systemic inflammatory response (C-reactive protein) measurement together with other clinicopathological variables were related to outcome. The second cohort consisted of patients in which pre-operative measurement of C-reactive protein was related to tumour proliferation (measured by Ki-67), local inflammatory reaction measured by infiltration of CD4+ and CD8+ and tumour associated macrophage (CD68+), microvessel density (CD34+) and COX-2 expression in the tumour tissue. The third cohort consisted of patients in whom C-reactive protein measurements were related to circulating cytokines/chemokines concentration involved in the regulation of the T-lymphocytes and other immune responses to the tumour.

The results of the first study were that on univariate analysis, stage ($p<0.001$), grade ($p<0.001$) and elevated C-reactive protein pre-operatively ($p<0.05$) and post-operatively ($p<0.01$) were significantly associated with cancer-specific survival. On multivariate analysis of patients who had a pre-operative C-reactive protein determination, stage ($p<0.008$), grade ($p<0.017$) and pre-operative C-reactive protein ($p<0.035$) were independently associated with cancer-specific survival. Those patients with an elevated pre-operative C-reactive protein concentration had a mean cancer-specific survival of 65.5 months compared with 103.7 months in those patients with a C-reactive protein concentration in the normal range ($\leq$10 mg/l). These findings would suggest that C-reactive protein is associated with disease progression and poor survival in patients with transitional cell carcinoma of the urinary bladder.

The results of the second study were that on univariate analysis, stratified by stage, C-reactive protein ($p<0.05$), increased Ki-67 labelling index ($p<0.05$), increased tumour associated macrophage ($p<0.01$), increased tumour microvessel density expression ($p<0.001$) increased COX-2 expression ($p<0.05$) and adjuvant therapy ($p<0.01$) were associated with poorer cancer-specific survival. On multivariate analysis

of these significant factors, stratified by stage, only C-reactive protein ($p=0.004$), increased tumour associated macrophage ($p<0.001$) and adjuvant therapy ($p<0.01$) were independently associated with poorer cancer-specific survival. These findings would suggest that tumour macrophage infiltration is an important local inflammatory response associated with disease progression and poor survival in patients with transitional cell carcinoma of the urinary bladder.

The results of the third study were that, using multiple cytokine analysis technology, Th0-type cytokines/chemokines (IL-6, IL-7, IL-8, IL-15 and GM-CSF), Th1-type cytokines/chemokines (IL-2, interferon-gamma and TNF-alpha) and Th2-type cytokines/chemokines (IL-4, IL-5, IL-10 and IL-13) measurement showed median plasma concentrations below the limit of detection (5pg/ml) irrespective of their C-reactive protein concentrations, stage and grade. However, macrophage/monocyte-type cytokines/chemokines (MIP-1 alpha, MIP-1 beta and MCP-1) and eosinophils-type cytokines/chemokines (EOTAXIN) had detectable median plasma concentrations but also were not associated with elevated C-reactive protein concentrations, stage and grade. These findings were inconclusive because of the low concentrations measured in the plasma. Further work is required to obtain reliable measurements in the plasma of patients with transitional cell carcinoma using multiple cytokine analysis technology.

Therefore, the results suggest that an elevated C-reactive protein concentration would be a useful marker of poor cancer-specific survival in patients with transitional cell carcinoma of the urinary bladder, in addition to tumour-based factors, and enable the stratification of patients for study and follow-up. Furthermore, that tumour macrophage infiltration is also an important predictor of outcome and is directly associated with C-reactive protein concentration and may be, through IL-6, an important mediator of C-reactive protein and the systemic inflammatory response.

1-INTRODUCTION

1.1 Incidence and mortality

Bladder cancer is the fourth most common cancer, after prostate, lung and colorectal cancer in men, accounting for 6% of all cancer cases, with 7,581 new cases diagnosed per year in men and a crude rate per 100,000 population of 26.5 for the UK population and 20 for Scotland. In women, it is the eighth most common cancer accounting for over 2% of all cancers with nearly 3,000 new cases diagnosed per year and a crude rate per 100,000 population of 10 for the UK population. In Scotland, nearly 500 men and 250 women were diagnosed with bladder cancer in 2001. There is a 1:30 risk of developing bladder cancer in men compared with 1:79 in women (Cancerstats, 2002).

Bladder cancer is rarely found incidentally at autopsy (Kishi et al, 1981) and the means by which it is diagnosed (cystoscopy and biopsy) have not changed for the last six decades. Therefore, it is unlikely that the increased incidence is due to technological developments or changes in health care practice.

Bladder cancer is the seventh most common cause of cancer death in UK men with nearly 3,200 deaths for the year 2003, and the tenth in women with 1,700 deaths for the year 2003. In Scotland, nearly 150 men and 100 women died due to bladder cancer. Eighty-six percent of male deaths and 90% of female deaths occur after the age of 65 years. Between 1979 and 2000, the mortality rates for 50-59 year old men fell by 55% and for women of the same age by 45% (Cancerstats, 2002).

Bladder cancer can occur in any age, even in children, but it is usually a disease of the middle-aged and elderly. The median age at diagnosis for transitional cell

carcinoma is 69 for men and 71 for women. However, the incidence increases directly with age and mortality is higher in the elderly (Cancerstat, 2002).

Survival of bladder cancer patients depends mostly on the depth of the tumour penetration in-to the bladder wall, in addition to other factors including multiple tumour foci, grade and tumour type. Patients with superficial disease (70%) have an excellent prognosis with 5-year survival rates between 80-90% compared with approximately 50% in patients with muscle-invasive disease (Zieger et al., 1998).

It is likely that the incidence of bladder cancer will continue to increase with the aging of the UK population. In contrast, there has been a decrease in mortality rate from bladder cancer. Whether the decrease in mortality is due to a fundamental change in the biology of the disease, alteration in risk factors for different types, better treatment, earlier diagnosis or a combination of all theses factors together remains uncertain.

1.2. Aetiology

Despite the identification of industrial teratogens and other carcinogens, the aetiology of bladder tumours in the majority of patients remains unexplained mainly due to the long latency period. This mechanism is multifactorial; it involves carcinoma-promoting factors (environmental influences, smoking, abuse of analgesics, genetic disposition) which support a malignant transformation that occurs in multiple steps.

1.2.1 Occupational Exposure

In Germany, in 1895, Ludwig Rehn showed the connection between papillary bladder tumours and an exposure to aniline dyes. In 1938, Hueper succeeded in proving the exogenous carcinogen: he showed that bladder carcinomas in dogs can be induced through exposure to beta-naphthylamine. More recently it has been estimated that approximately 35% of bladder cancers in men have an occupational origin, in occupations in which chemicals of the aryl hydrocarbon group are used (Cole et al 1972). These include benzedrine, α- and β-napthylamines, aminobipenyl, and orthotolidine, with a long latent period of between 30-50 years (Case et al, 1954).

Other occupations reported to be associated with an increased risk of bladder cancer include those of autoworkers, painters, truck drivers, drill press operators, leather workers, metal workers and machinists; also, those occupations involved with organic chemicals such as dry cleaners, paper manufactures, rope and twine workers, dental technicians, barbers and beauticians, physicians, workers in apparel manufacturing and plumbers (Morrison, 1984).

The connection between the development of bladder carcinoma and individual genetic disposition of the enzymes N-acetyltransferase-2, (which, among other things, converts aromatic amines, drugs and caffeine) is under debate. There are two so called

human phenotypes: the fast and slow acetylator. In slow acetylation of the oxidative metabolism, for example, of aromatic amines, there are many steps in which aryl nitrenium ions are produced that allow bladder tumours to occur. The percentage of each phenotype is markedly varied in different ethnic groups. In Europe, 50-60% are slow acetylators, whereas in China the number is 7.6% (Golka et al., 2002).

1.2.2 Cigarette smoking

Zeegers and associates performed a meta-analysis of 43 epidemiologic studies on smoking characteristics for bladder cancer risk and concluded that current cigarette smokers have an approximately threefold higher risk of urinary tract cancers than non-smokers (Zeegers et al., 2004). Although tobacco smoke condensates contain a mixture of chemicals, the molecular mechanisms are likely to be based on exposure to the carcinogens 4-aminobiphenyl and 2-naphthylamine in cigarette smoke. The risk is associated with the number of cigarettes smoked, the duration of smoking, and degree of inhalation. Ex-smokers have reduced incidence of bladder cancer compared with the active smoker (Bryant et al., 1988). The reduction of the risk to that of the non-smoking population takes about 20 years after stopping smoking. This is far longer compared to the reduction in risk of cardiovascular disease and lung cancer.

p53 gene mutations are related to tobacco-smoke carcinogens and the frequency of smoking increases the extent of DNA damage (Bernardini et al.,2001).

1.2.3 Age and sex

The male: female ratio of age-standardised rates fell from 4.0:1.0 in 1975 to 3.5:1.0 in 2001. This discrepancy between the genders in the numbers of new cases of bladder cancer is perhaps not surprising because during the past 30 years women have joined the male workplace and have changed habits that have exposed them to both industrial and environmental carcinogens (Edward et al 1997).

1.2.4 Race

According to the SEER data from the United States, African Americans, especially women, have a 60 to 100% higher mortality from bladder cancer than do their white counterparts. Several explanations for the higher mortality among such racial differences are: more aggressive disease, low socio-economic status, elapsed time from symptoms to onset of treatment, occupational exposure, smoking and difference in metabolism of toxic substances (Madeb and Messing, 2004).

1.2.5 Chronic cystitis and other infections

Cystitis-induced bladder cancer is usually associated with severe, long-term infection. Chronic cystitis due to the presence of indwelling catheter or calculi is associated with an increased risk for bladder cancer. Delnay and co-workers studied 208 patients with paraplegia with long-term catheter with 7 years follow-up and found that a total of 17 patients were identified with malignancy, including 10 with squamous cell carcinoma, 5 with transitional cell carcinoma and 2 with adenocarcinoma (Delnay et al.,1995).

Infestation of the bladder with Schistasoma haematobium also appears to be related to the development of bladder cancer, often squamous cell carcinoma. In Egypt,

where schistomaiasis is endemic among men, squamous cell carcinoma of the bladder (bilharzias bladder cancer) is the most common malignancy (Badawi et al., 1995). Viral infection has also been blamed in relation to bladder cancer, LaRue and co-workers (1995) reported that as many as 35% of bladder tumours have evidence of the human papillomavirus HPV. However, recent studies suggest that HPV is unlikely to play a major role in the development of bladder TCC (Youshya et al., 2005).

The mechanism of such carcinogenicity is most likely related to the formation of nitrate and N-nitroso compounds in the bladder from microbial metabolism of normal urinary constitution (Delnay et al., 1995).

1.2.6 Heredity

No epidemiologic evidence exists for a hereditary cause of bladder cancer, although familial clusters of bladder cancer have been reported. Of these, lynch syndrome II, an inherited syndrome associated with colorectal cancer without polyposis and with extra-colonic cancer site, has been described in which four male siblings developed transitional cell carcinoma of the urinary bladder (Lynch et al, 1990)

Other reported cases of bladder cancer have been attributed to pelvic irradiation (Sella et al, 1989) and cyclophosphamide (Tuttle et al, 1988).

1.3 Pathology

1.3.1 Tumour staging

Transitional cell carcinoma has been traditionally characterised as either superficial or invasive. Superficial tumours are the most common initial presentation (70 to 75%; Heney, 1992). However, there are 3 stages of superficial disease. These include "flat" carcinoma in situ confined to the urothelium, papillary tumours confined to the mucosa (Ta) and papillary or nodular tumours with invasion into the lamina propria (T1).

Carcinoma in situ (Tis) replaces the urothelial layer with high-grade, anaplastic cells and appears as flat red patches in the bladder and may be difficult to distinguish from the changes of chronic infection or inflammation (Witjes, 2004). Data from the literature suggest that these lesions have high recurrence rates, a high likelihood of invasion, and are associated with poor cancer survival (Eble et al., 2004). These lesions are frequently multifocal and may present with other papillary lesions. Urinary cytology study is useful in detecting non-visible lesions because the exfoliated cells in these cases exhibit severe dysplasia.

Stage Ta lesions account for approximately 70% of superficial TCC. Histologically, these papillary tumours are composed of a branching fibrovascular core and a mucosa greater than 8 cell layers with features of anaplasia. Ta lesions are typically low-grade.

Stage T1 bladder carcinoma has demonstrated lamina propria invasion. They have a high percentage of progression to more advanced disease and tend to be high-

grade tumours (Heney, 1992). The malignant potential of these lesions separates them clearly from Ta and carcinoma in situ lesions. Many have argued that these tumours should not be considered "superficial" because they demonstrate a natural history more consistent with muscle-invasive cancer especially if they have a high-grade G3, since, many patients with pathologic T1 carcinoma treated with bacille Calmette-Guérin (BCG) will still progress to muscle-invasive disease (Manoharan and Soloway, 2005). Further stage subdivision has been proposed for T1 tumours according to the depth of lamina propria invasion (Figure 1.1).

Currently, pathologic staging of bladder cancer is usually carried out according to the 2002 American Joint Committee on Cancer/International Union Against Cancer TNM staging guidelines (Eble et al., 2004).

TNM staging system for bladder carcinoma (6^{th} edition)

Primary tumour (T)

The suffix (m) is added to the appropriate category to indicate multiple tumours and the suffix (is) to indicate associated carcinoma in situ.

TX Primary tumour cannot be assessed

T0 No evidence of primary tumour

Ta Non-invasive papillary carcinoma

Tis Carcinoma in situ "flat tumour"

T1 Tumour invades subepithelial connective tissue

T2 Tumour invades muscle

T2a tumour invades superficial muscle (inner half)

T2b Tumour invades deep muscle (outer half)

T3 Tumour invades perivesical tissue

T3a microscopically

T3b macroscopically

T4 Tumour invades any of the following: prostate, uterus, vagina, pelvic wall, and abdominal wall

T4a Tumour invades prostate, uterus or vagina.

T4b Tumour invades pelvic wall or abdominal wall.

Regional lymph nodes (N)

Nx Cannot be assessed

N0 No metastasis in regional node

N1 Metastasis in one node 2cm or less in diameter

N2 Metastasis in one node 2-5 cm in diameter or multiple nodes not > 5 cm in diameter.

N3 Metastasis in one node > 5 cm in diameter

Distal metastasis (M)

Mx Cannot be assessed

M0 No distal metastasis

M1 Distal metastasis

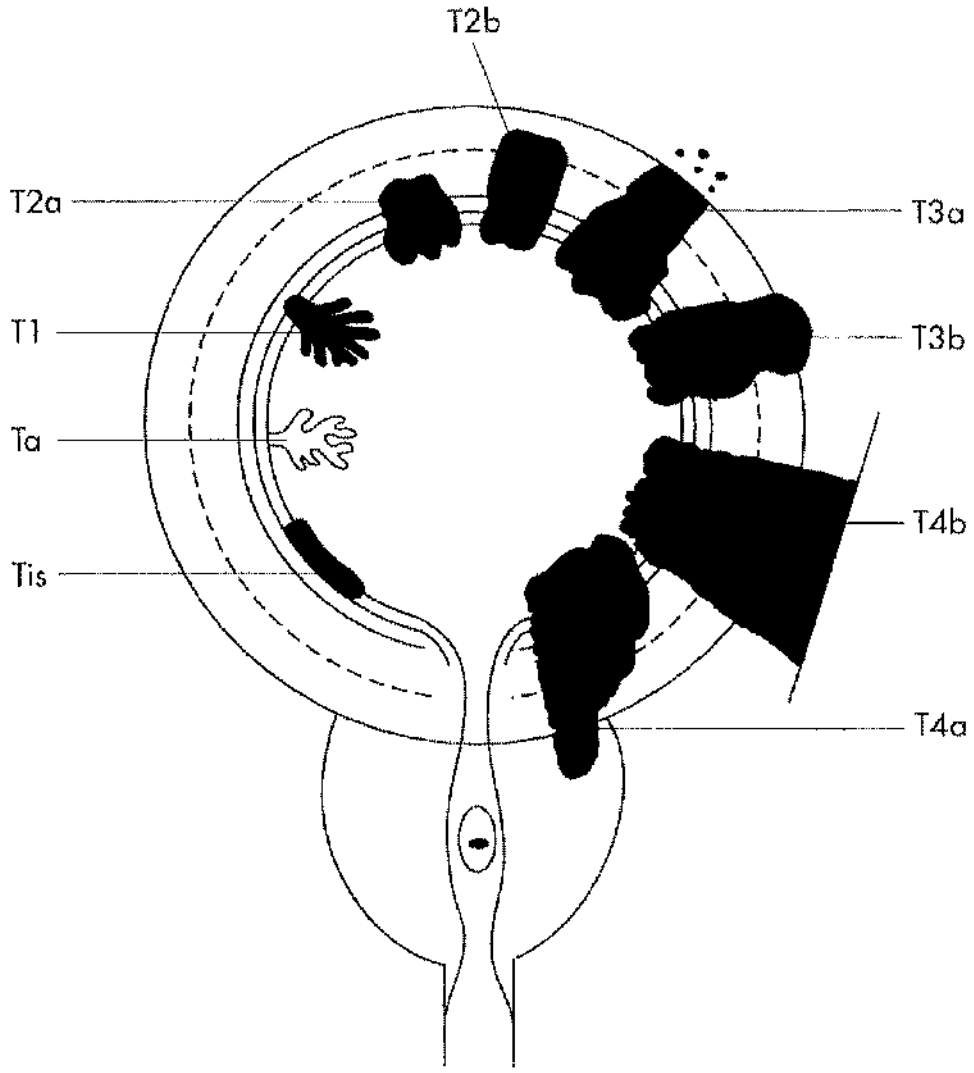

Figure 1.1 T staging of bladder cancer

Tis, carcinoma in situ; Ta, tumour involves mucosa only; T1, tumour invades through lamina propria; T2a, tumour invades into superficial detrusor muscle; T2b, tumour invades into deep detrusor muscle; T3a, tumour microscopically invades perivesical tissue; T3b, tumour macroscopically invades perivesical tissue and forms extravesical mass; T4a, tumour invading prostate/uterus/vagina; T4b, tumour fixed to pelvic wall (adapted from Colquhoun and Mellon, 2002).

1.3.2 Tumour grading

Urinary bladder tumours are graded traditionally as well-differentiated (G1), moderately-differentiated (G2), or poorly-differentiated (G3) according to the degree of cellular anaplasia (Busch et al., 2002). There is a strong correlation between tumour stage and grade. Most well-differentiated tumours are superficial and most poorly-differentiated tumours are invasive.

Considerable disagreement has been shown in the interpretation of histological grade between pathologists (Humphrey, 2004). Tumour grading is such a controversial issue that the initial grading in 1974 was replaced with the current version in 1999. Furthermore, the new 2004 grading system could help to narrow the differences especially in G2 tumours. The chief difference in this latest grading system compared with the WHO 1999 scheme is that rather than three grades of papillary urothelial carcinoma, there are two (low-grade and high-grade) in the WHO 2004 classification. The authors of the WHO 2004 system cite genetic studies that suggest there are "two major subtypes of urothelial neoplasms which might have a distinctly different clinical course": genetically stable tumours that include most non-invasive low-grade carcinomas and genetically unstable non-invasive high-grade carcinomas and invasive carcinomas. Currently, these genetic differences and specific molecular markers, such as cytokeratin 20, p53, p63, CD44, FGFR3, and Ki-67 hold promise for the future but are not in routine use in grading of urothelial proliferations (Eble et al, 2004, Humphrey, 2004).

1.4 Clinical Features of transitional cell carcinoma of the urinary bladder

Early symptom recognition in bladder tumour is a key to better prognosis. The most common sign of bladder cancer is haematuria. Approximately 80% of all patients with TCC will be diagnosed with either gross or microscopic haematuria. In one study of 1000 consecutive patients evaluated for gross painless haematuria, 15% were found to have bladder cancer (Turner et al., 1977). Microscopic haematuria (usually asymptomatic) is a much more common presenting symptom than gross haematuria. Previous studies have shown that between 2 to 22% of patients with microscopic haematuria will be found to have a malignancy (Carson et al., 1979). Other presenting symptoms are due to bladder irritability with dysuria, frequency, and urgency being seen in around 20% of patients. In these instances the patients will often have invasive lesions or CIS (Farrow et al., 1977). Other patients may present with recurrent urinary tract infections, loin pain secondary to an obstructed ureter, sterile pyuria, or as an incidental finding at cystoscopy. Symptoms of advanced disease include bone pain from bone metastasis or flank pain from retroperitoneal metastasis or urethral obstruction.

In the majority of patients, clinical examination will usually be normal due to the superficial nature of the disease. Occasionally in advanced disease an abdominal or pelvic mass may be palpable. Hepatomegaly, lymphodema from occlusive pelvic lymphadenopathy and supraclavicular lymphadenopathy are signs of metastatic disease (Walsh et al., 2002).

Transitional cell carcinomas have a tendency to recur after excision (Figure 1.2); such recurrence could be similar to the initial tumour or the tumour could progress to more advanced tumour (grade and stage). Although the term "recurrence" is used, most of the subsequent tumours arise at different sites from the original lesion and the time

interval could be many years. Recurrent tumours reflect new tumours in some cases, and in other instances, they share the same clonal abnormalities as the initial tumour and represent a true recurrence of the initial lesion as a result of shedding and implantation of the original tumour cells (Colquhoun and Mellon, 2002).

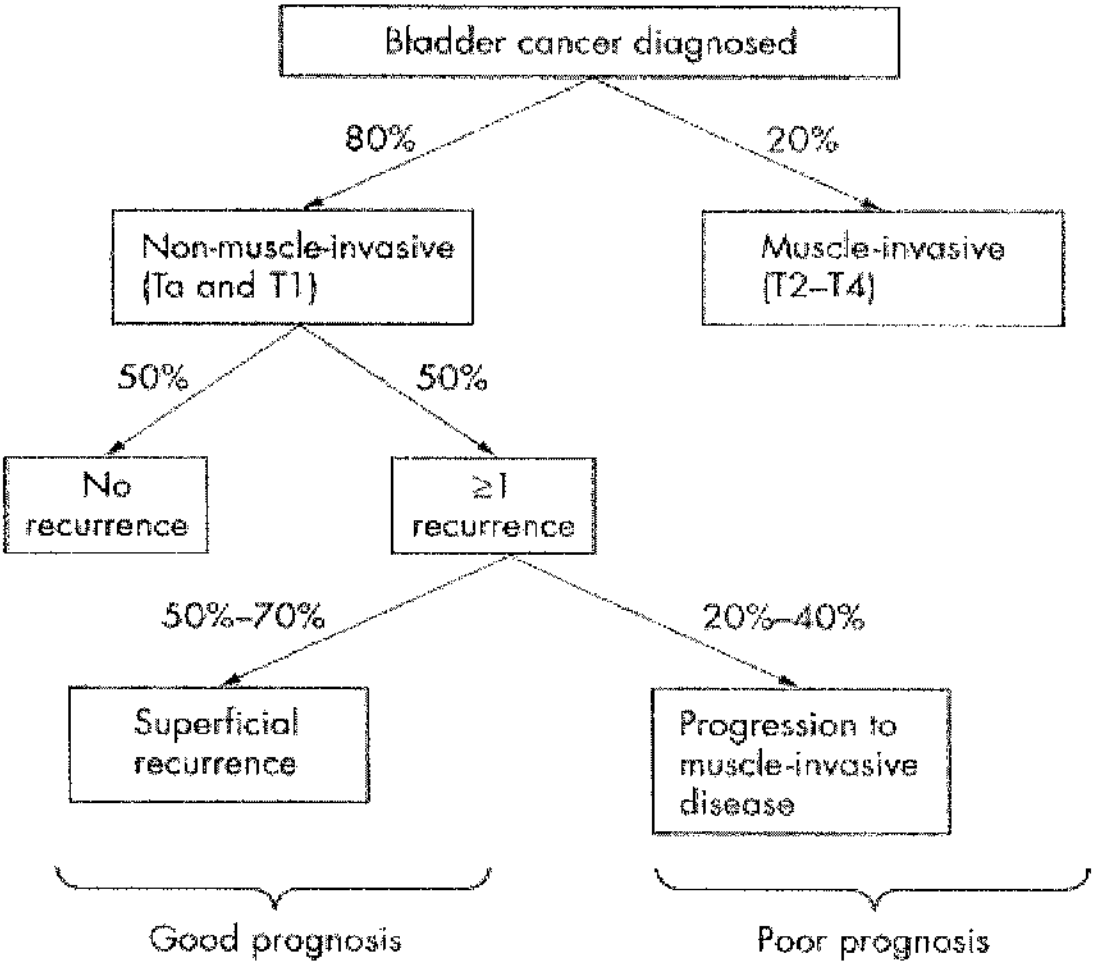

Figure 1.2 The natural history of bladder cancer (adapted from Colquhoun and Mellon, 2002)

1.5 Treatment of transitional cell carcinoma of the urinary bladder

All patients with bladder tumours should initially undergo transurethral resection of the tumour. A bimanual examination of the bladder should be performed both before and after the resection in order to stage the tumour clinically. Random biopsies of apparently normal bladder distant from the tumour can be taken to look for the presence of associated carcinoma in situ. Further management is then largely based on the stage and grade of the tumour.

1.5.1 Superficial disease

Management of patients with Ta and T1 tumour will take into account the risk of recurrence and progression, side effects and cost-effectiveness. pTa tumours, comprise 35% of all bladder cancers and are usually well-differentiated (G1). Less than 5% of pTa tumours progress to invasive disease and the 10 year survival of around 96%. In the United Kingdom pT1 tumours comprise approximately 30% of bladder cancers. Prognosis is slightly worse than with pTa tumours, with a 10 year survival of around 65% (Zieger et al., 1998). Using the above parameters, superficial bladder tumour can be divided into:

- Low-risk tumours: single, Ta, G1, ≤ 3 cm diameter.
- Intermediate: Ta-1, G1-2, multimodal, >3 cm diameter, all other tumours.
- High-risk tumours: T1, G3, multimodal or highly recurrent, CIS (TIS).

For the low-risk tumours, treatment consists of intravesical chemotherapy post-TURBT: a single dose of intravesical instillation of mitomycin or epirubicin within 24 hours post-tumour resection has been found to reduce recurrence rate by approximately 50%. In intermediate risk tumours a further instillation is required. It is recommended that tumours with a higher risk of recurrence should be treated with a 4–8 week course

of bladder instillations. In the case of carcinoma in situ or multiple recurrences it is recommended intravesical BCG therapy be given (Alexanrdroff et al., 1999). . BCG therapy has been found to be effective in preventing disease progression and reducing the number of tumour recurrence ((Sylvester et al., 2002, Han and Pan, 2006).

1.5.2 Invasive bladder cancer

Invasive bladder cancer carries a poor prognosis with transurethral resection alone; the 5 year survival is less than 50%. However, if patients are carefully selected for treatment, 10 year survival can reach up to 75% (Walsh et al., 2002). Tumours should be ideally solitary, papillary, less than 3 cm in size, and a biopsy of the bladder wall deep to the tumour must be clear if this approach is adopted. Since this does not apply in most cases, a more aggressive approach is necessary including either external beam radiation or cystectomy with urinary diversion or a neobladder construction.

1.5.3 Disseminated disease

Distant metastases are mainly located in liver (approximately 40%), lung (approximately 40%) and bone (approximately 20%). More than 50% of all patients with invasive tumours also have distant metastases (Walsh et al., 2002). The treatment of disseminated bladder cancer is essentially palliative as patients have a median survival of only 6 months. Multi-agent chemotherapy using methotrexate, vinblastine, docorubicin and cisplatin (M-VAC) can double the median survival with approximately 15% of patients surviving 2 years, but it carries a significant morbidity in patients who may already be very weak (Roberts, 2005).

1.5.4 Follow-up after TURB

A superficial bladder tumour has a high tendency to recur after resection (Figure 1.2). Several causes have been suggested for this high recurrence rate: incomplete resection, implantation at traumatised sites in the bladder or rapid growth of epithelial malignancy. Therefore, an early check cystoscopy preferably under general anaesthetic 3 months after resection is now usual in almost all cases of superficial bladder cancer. The findings at the initial 3 month check cystoscopy give a useful guide to prognosis. If no recurrence is identified at 3 months, approximately 80% of patients will have no further recurrence. On the other hand, of those patients noted to have a recurrence at 3 months, approximatly10% will have no further recurrence (Ali-El-Dein et al., 2003).

The frequency of later cystoscopies should be adapted to the prognostic factors of the tumour. In low-risk tumours with no recurrence at 3 months, a follow-up cystoscopy can be delayed until 9 months later and then yearly up to 5 years. In contrast, patients with high-risk tumours, a cystoscopy every 3 months during the first 2 years remains the most commonly adopted follow-up schedule (Hall et al., 1994). Cystoscopy should then follow every 4 months until the third year, every 6 months thereafter for up to 5 years and then yearly. The schedule of follow-up in the intermediate group lies in between. With any recurrence, the schedule of cystoscopies is re-started from the beginning (Hall et al., 1994).

1.6 Immunotherapy

Immunological treatment for bladder cancer falls into two general categories: passive and active. Passive immunotherapy involves the direct administration of molecules or cells to a patient and requires no direct involvement of the patient immune system. Active immunotherapy attempts to enhance the host immune response. Passive and active immunotherapy may be non-specific or specific in nature. Non-specific immunotherapy induces inflammation or otherwise amplifies an already present immune response, e.g. bacillus Calmette-Guerin (BCG). In contrast, specific immunotherapy requires tumour specific antigen recognition by T cells and/or antibodies (Walsh et al., 2002).

Passive immunotherapy: non-specific, consists of the administration of cytokines that indirectly affect tumour growth by stimulating cytotoxic T cells or by acting directly on tumour cells e.g. IFN gamma against superficial bladder cancer (Giannopoulos et al, 2003). On the other hand, specific immunotherapy, target tumour antigen-specific T-lymphocytes or antibodies produced by B-lymphocytes are adoptively transferred into a recipient. Herceptin, the mAb specific for HER-2/neu used in the treatment of breast cancer, has recently been studied in bladder cancer (Latif et al, 2004, Hauser-Kronberger et al., 2006). Active immunotherapy: non-specific, which stimulates the innate inflammatory response, this consists of mainly BCG and cytokines. Active specific immunotherapy is the vaccination of patients to induce long-lived, tumour-specific immunity.

1.6.1 BCG

BCG is an attenuated live strain of mycobacterium bovis. In 1976, Morales, Eidinger and Bruce were the first to report on successful treatment of superficial bladder

cancer with BCG (Morales et al, 1976). Since then, BCG has become the treatment of choice for high-risk superficial bladder tumour. However, the mode of action has not been fully understood and it is believed to be immunological rather than a direct cytotoxic effect in that it activates macrophages and dendritic cells (Alexandroff et al., 1999).

Wide acceptance of BCG immunotherapy for bladder cancer, however, did not begin until the controlled study by the Southwest Oncology Group showed in 1980 that there were unequivocal benefits in terms of decreased recurrence rate and increased median time to recurrence in patients given BCG immuno-prophylaxis after local surgery (Lamm et al., 1980). A recent meta-analysis of randomised clinical trials, investigating the effect of intravesical BCG and progression showed that BCG immunotherapy significantly reduced the risk of progression after transurethral resection in patients with superficial disease who receive maintenance treatment (Sylvester et al., 2002). The authors suggest that BCG is the agent of choice for patients with intermediate and high-risk papillary tumours and those with carcinoma in situ. More recently, Han and Pan (2006) in meta-analysis of randomised clinical trials concluded that intravesical BCG with maintenance treatment is effective for the prophylaxis of tumour recurrence in superficial bladder cancer. Furthermore, for patients with papillary carcinoma, they concluded that adjuvant intravesical BCG with maintenance therapy should be offered as the treatment of choice.

Standard treatment consists of BCG instillations given over a 6 week period. Complete remission is obtained in up to 70% of cases and half of patients will be disease-free at 5 years. Side effects of treatment with BCG are common, with about half the patients experiencing an increase in frequency and dysuria. Maintenance therapy

with booster cycles up to 36 months is advocated to prevent recurrence (Huncharek and Kupelnick, 2003).

1.6.2 Cytokines

Interferons (IFN): IFNs are host-produced glycoprotein in response to antigenic stimuli and have multiple anti-tumour activities that act to mediate immune responses through antiviral, anti-proliferative and regulatory activities. The combination of interferon-alpha with bacillus Calmette-Guerin appears to result in remarkable synergy, affecting not only bladder tumours directly but also enhancing the immune response to bacillus Calmette-Guerin by orders of magnitude (O'Donnell, 2003). A recent national multi-centre phase II trial of the combination of BCG and interferon in 1,007 patients showed that although BCG plus IFN-alpha can be effective in both patients naive to BCG and those having BCG failure, certain patient and tumour characteristics influence durable response (Joudi et al., 2006).

Intravesical IL-2 has shown promise in the treatment of superficial bladder cancer (Den Otter et al., 1998). IL-12 has been shown to induce T-lymphocyte and natural killer cell proliferation/activation and production of IFN gamma. A phase I study has evaluated intravesical recombinant human IL-12 administration in patients with superficial bladder cancer (Weiss et al., 2003).

1.6.3 COX-2 inhibitors

Mohammed and co-workers examined the potential anti-tumour effects of a COX-2 inhibitor in the canine model with invasive bladder cancer. All tumours positively immunostained for COX-2, and COX-2 inhibition therapy resulted in a statistically significant reduction in tumour volume in 12 of 18 animals, with an

associated increased rate of apoptosis and decrease in PGE2 (Mohammed et al., 2002). The clinical study of COX-2 inhibitors in bladder cancer is just underway, the BOXIT (Bladder COX-2 Inhibition Trial), which is a randomised phase III parallel-group multi-centre double-blind placebo-controlled clinical trial in patients with transitional cell carcinoma of the bladder by giving Celecoxib for 2 years in addition to their standard treatment and assessing outcome after 3 years (Cancer Research UK).

1.6.4 Other immunotherapy

Bropirimine is an immuno-modulating agent considered to boost immunity in bladder cancer. Oral bropirimine has been shown to have anti-cancer activity in carcinoma in situ of bladder cancer. In a phase III European clinical study comparing 55 BCG-naive patients randomised to either oral bropirimine (n=27) or BCG (n=28), the complete response rate was 92% and 100% with a mean duration of 12.6 months and 12.3 months respectively (Witjes et al., 1999).

Keyhole-Limpet Hemocyanin (KLH) is an antigenic protein from the hemolymph of *Megathura crenulata* and a non-specific immuno-modulator, has shown efficacy in Ta and T1 bladder cancer and CIS. Although less effective than BCG, it is less toxic and may have a role as an alternative biologic response modifier in treating superficial bladder cancer (Walsh et al., 2002).

1.7 Tumour growth and spread

There is considerable complexity with the process of tumour growth and metastasis. Not only tumour-based factors (intrinsic) but also host-based factors (extrinsic) play a role in this process. Although biomarkers can reflect activity in individual pathways, when a global assessment is made of tumour growth then single markers are of limited value. Carcinogenesis is currently considered to be divided into initiation, promotion and progression (Walsh et al., 2002).

1.7.1 Tumour-based factors (intrinsic)

The ability of tumour cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of apoptosis (programmed cell death). It is believed that acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer (Hanahan and Weinberg, 2000). The cellular homeostasis is defined by a balance between the destructive and proliferative processes of cells. Cell death serves as a local defence mechanism to remove potentially harmful damaged cells, caused by cytotoxic inflammatory response and is a lifelong programme that genetically regulates the cell destruction process.

Apoptosis appears to be triggered through receptor-ligand interaction, mainly the fas-fas legand by binding of the Fas-ligand expressed on the cytotoxic T-lymphocyte to Fas expressed on the target cell. This will trigger a cascade of changes and Fas interacts with proteins in the death pathway leading to activation of the caspase enzyme. This in turn activates caspase-activated DNAse which migrates into the nucleus leading to chromosome de-fragmentation and consequently a complete loss of the cell.

In a recent study of 147 patients with superficial bladder cancers the apoptotic index (the number of apoptotic cells divided by the total number of tumour cell population) and Ki-67 labelling index were assessed. The apoptotic index and Ki-67 labelling index appear to correlate with tumour grade and stage. In addition, apoptotic index appears to correlate with tumour size and grade (Gonzalez-Campora et al., 2006).

The behaviour of bladder cancer to date is not predictable and there has been a huge interest in identifying the molecular characteristics that determine a tumour's behaviour, both to determine the prognosis of an individual tumour and to identify new therapeutic targets. There are clinical-pathological markers such as tumour stage (Able et al., 1988), grade of anaplasia (Pauwells et al., 1988), tumour size (Hellsten et al., 1983, multiplicity (Pocock et al., 1982) and growth pattern (Kern, 1984).

Recently, the European Organisation for Research and Treatment of Cancer (EORTC) analysed the results collected over 30 years of 2596 patients with superficial bladder cancer (Tis, Ta, T1) with a median follow-up of 3.9 years and a maximum follow-up of 14.8 years. 1240 (47.8%) had recurrence 313 (12.1%) in the first 3 months, with progression amongst those with recurrence of 279 (10.7%). During the follow-up period 853 (32.9%) died and of these 262 (10.1%) were bladder cancer-related. On multivariate analysis, prior recurrence, number of tumours, tumour size, T stage (Tis, Ta, T1) and grade (G1, G2, G3) were all associated with tumour recurrence and progression. The most important of the prognostic factors for progression were the tumour stage, grade, and the presence of carcinoma in situ which reflect the biological aggressiveness of the disease. The authors also suggest that the molecular markers such as p53, Ki-67 labelling index and COX-2 have some promise; however, they concluded that these have not been sufficiently validated for routine use at this time (Sylvester et al., 2006).

Potential tumour-based prognostic factors that can be classified into the following groups: proto-oncogenes/oncogenes, tumour suppressor genes, cell cycle regulators and angiogenesis-related factors (Figure 1.3).

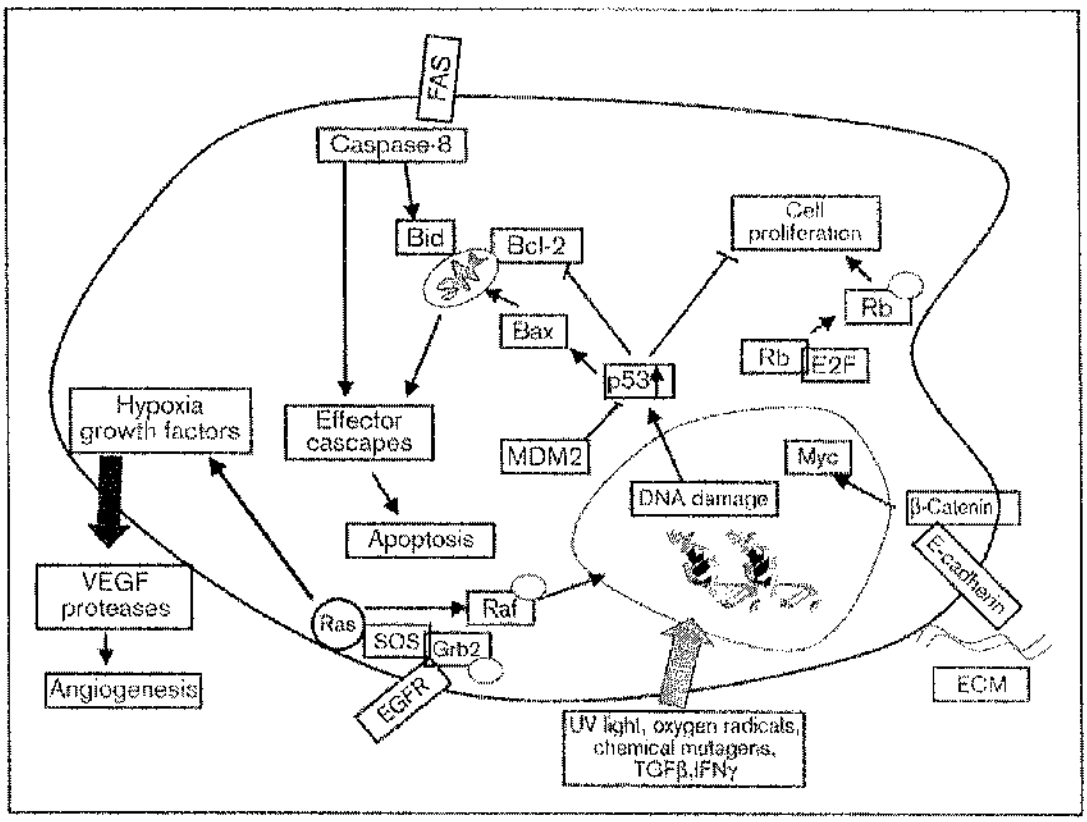

Figure 1.3 The complex oncogenic pathways in the established cancer cell. (Adapted from Duggan et al., 2004)

EGFR, epithelial growth factor receptor; ECM, extracellular matrix; VEGF, vascular endothelial growth factor; TGF, transforming growth factor; IFN, interferon.

Proto-oncogenes/oncogenes

Oncogenes are altered normal genes that encode for malignant phenotype, primarily by permitting cells to escape from normal mechanism of growth control.

Pathological expression of EGFR leads to uncontrolled cell proliferation through increased angiogenesis and reduced apoptosis. However, the mechanism by which EGFR expression is associated with poor prognosis is not entirely clear, although there is some evidence linking EGFR-stimulated activation of activator protein-1 transcription factor with induction of matrix metalloproteinase activity (Wells, 2000). Several investigators have shown a positive association between over-expression of epidermal growth factor receptor (EGFR) and high-grade, high-stage bladder cancer and poor survival (Neal et al., 1990, Lipponen et al., 1994).

Tumour suppressor genes

These are genes responsible for coding for proteins that regulate cell growth, DNA repair or apoptosis. Deletion or inactivation of these tumour suppressor genes could encourage unregulated growth or failure to direct DNA-damaged cells to programmed death. This will lead to uncontrolled proliferation of genetically altered clones. Three suppressor gene loci are found to be associated with bladder cancer; they are p53 (on chromosome 17p), the retinoblastoma gene Rb gene, (on chromosome 13q) and the gene on chromosome 9 at p15 and p16.

P53 is the most frequent altered gene in human cancer (Hall and McCluggage, 2006). The normal p53 protein (wt p53) is located on the 17p 13,1 locus and has several functions; acting as transcription factor that suppresses cell proliferation and directing the DNA-damaged cell toward apoptosis before DNA replication S-phase of cell cycle occurs. P53 mutation has been associated with genomic instability leading to progressive development of further mutation. Bladder cancers with p53 abnormalities appear to behave more aggressively (Cordon-Cordo, 1995). T1 tumours that express wild-type (normal) p53 are reported to have a progression rate of around 2.5% per year

compared with 20 % per year in tumours expressing mutant p53. However, a meta-analysis by Schmitz-Dräger and co-workers showed that approximately half of the studies, which include p53 in multivariate analyses, confirmed its value as a prognostic marker of progression in T1 bladder cancer. In addition, in only 2 of the 7 studies in muscle-invasive bladder cancer was p53 confirmed as an independent prognostic marker of disease progression (Schmitz-Dräger et al., 2000). Indeed, a recent meta-analysis concluded that the evidence is not sufficient to routinely use p53 as a marker of outcome in patients with bladder cancer (Malats et al., 2005).

Cell cycle regulators

The expression of the human Ki-67 protein is strictly associated with cell proliferation. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The fact that the Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2 and mitosis), but is absent from resting cells (G0), makes it an excellent marker for determining the so called growth fraction of a given cell population. Little is known about the function of this protein. However, we do know that it is vital for cell proliferation, since removal of pKi-67 using antisense nucleotides prevents cell proliferation (Schluter et al., 1993).

A number of studies have shown that Ki-67 is an independent prognostic marker of bladder cancer progression and recurrence in multivariate analysis. For example, in 159 patients with Ta or T1 bladder cancer, a high Ki-67 index (≥18%) and multifocality were independently associated with increased recurrence, progression and poor survival (Santos et al., 2003). Similarly, a study of 114 patients with bladder cancer showed that Ki-67 expression was an independent prognostic factor associated with poor disease-

specific survival in multivariate analysis (Popov et al., 1997). These studies consistently indicate that elevated Ki-67 expression is a promising marker for increased recurrence and progression in superficial bladder cancer.

A study for p21 and p53 expression in bladder cancer patients showed that p21 labelling was an independent predictor of cancer survival (Chatterjee et al 2004). In 120 consecutive cases of urothelial carcinoma, p27 levels were significantly higher in low-grade, superficial, papillary, and slowly-proliferating tumours (Korkolopoulou et al., 2000).

Cyclin D1 is a positive regulator of the cell cycle. In a multicenter study of 207 patients with superficial (Ta or T1) bladder cancer, the cyclin D1 expression level was an independent predictor of tumour recurrence. However, it did not provide additional prognostic information on disease progression (Liukkonen et al., 2000). A study using a tissue micro-array of 2317 specimens from 1842 patients with bladder cancer staged Ta through to T3 demonstrated that low cyclin E expression was associated with poor overall survival in all patients but it had no prognostic impact independent of stage (Richter et al., 2000)

Angiogenesis-related factors

Cyclooxygenases (COX) are enzymes that are responsible for formation of prostanoids (including prostaglandins, prostacyclin and thromboxane). COX enzyme converts arachidonic acid to prostaglandin H_2, the precursor of the series-2 prostanoids (Table 1.1). These are among the most important enzymes involved in the regulation of the immune response and play a key role in angiogenesis, the inhibition of apoptosis, and cell proliferation and motility (Figure 1.4).

	Cyclo-oxygenase-1	Cyclo-oxygenase-2
Expression	Constitutive	Constitutively expressed only in brain, lung, kidney
	Found in all tissues	Rapidly induced in inflammatory tissue (1-3 h)
		Induced by cytokines and growth factors (IL-2,TNF-α)
Functions	Housekeeping function in platelet, stomach, kidneys, and endothelium	Inflammatory process in macrophages, leucocytes, fibroblasts, and endothelium
Inhibition	Aspirin and non-steroidal anti-inflammatory drugs	Anti-inflammatory cytokines (IL-10), Aspirin, non-steroidal anti-inflammatory drugs and glucocorticoids.

Table 1.1 The function and production of cyclooxygenase enzymes (Adapted from Bjorkman, 1998)

Figure 1.4 shows the interactive relationship among cancer cells, endothelial cells and infiltrating inflammatory cells at the site of tumour. The prostaglandin pool is contributed to by all three different cell types and occasionally stromal cells. The positive feedback through prostaglandin receptors increases COX-2 expression and ensures the continued generation of prostaglandins. In the cancer cell, prostaglandin signalling also results in the production of multiple angiogenesis factors, through which they stimulate neovascular formation. In inflammatory cells, prostaglandin signalling stimulates the generation of pro-inflammatory molecules such as IL-2, which further recruits additional circulating monocytes and amplifies the inflammatory response. As a

response to the increased levels of prostaglandins, angiogenesis factors and pro-inflammatory molecules are believed to lead to endothelial cell proliferation, migration and stimulation of vessel formation. These will provide additional nutrients for oncogenesis as well as a potential route for metastasis (Zha et al., 2004).

VEGF expression determined by in situ hybridisation was an independent prognostic factor for disease recurrence by multivariate analysis in 55 patients with muscle-invasive bladder cancer treated with neoadjuvant MVAC chemotherapy and radical cystectomy (Inoue et al. 2000).

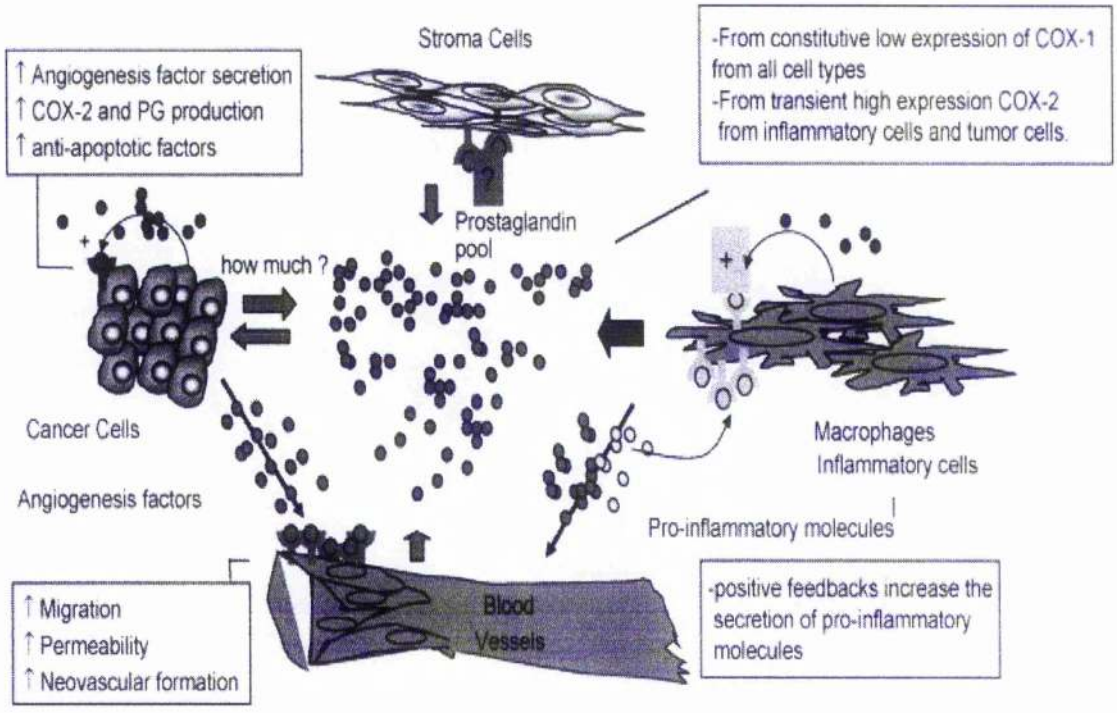

Figure 1.4 COX-2 in angiogenesis. (Adapted from Zha et al., 2004)

1.7.3 Host factors determining progression in bladder cancer (extrinsic factors)

The host response to the changes from normal cell to tumour cell is complex and crucial to tumour progression. Such a response appears to either act to kill the tumour cells or to enable the tumour cells to grow and spread.

Immune Surveillance

Immune surveillance explains the mechanism by which the immune system keeps cancers from arising. The theory states that patrolling cells of the immune system provide continuing body-wide surveillance and eliminate foreign cells or cells that undergo cancerous transformation. This theory developed by a number of workers including Thomas (1959) and Burnet (1970) is consistent with a number of clinical observations. For example, cancer is rare in children and young adults, but appears more frequently with advancing age, when the efficiency of the immune system declines. In addition, most solid tumours were found to contain large numbers of lymphocytes, suggesting that the cellular immune system recognised the malignant neoplasm as foreign and attacked it (Algarra et al., 2004).

The connection between tumour growth and immunity is not new; in fact Virchow in 1863 noted leucocytes in neoplastic tissues and made a connection between inflammation and cancer. He suggested that the "lymphoreticular infiltrate" reflected the origin of cancer at sites of chronic inflammation (Balkwill and Mantovani, 2001). In 1909 Ehrlich showed that tumours transplanted to non-tumour bearing mice initially would grow, then shrink and disappear. He won the Nobel Prize in 1908 for his suggestion that antibodies could be used like "magic bullets" to treat all types of diseases!

More recent observations also supporting the immune surveillance theory include the presence of tumour antigens and promotion of tumour growth when the thymus is removed or when immunosuppressive drugs are given (Baniyash, 2006).

The immune system recognises tumours and demonstrates its response to tumour antigens. Such recognition appears to be through Human Leucocyte Antigens (HLA). The Human Leucocyte Antigens (HLA) are a group of genes in the human major histocompatibility complex (MHC) on human chromosome 6 that encodes the cell-surface antigen-presenting proteins. Every organism has two sets of HLA genes, HLA class I and II. HLA class I molecules are found on almost every nucleated cell of the body while HLA class II molecules are found only on a few specialised cell types, including macrophages, dendritic cells and activated lymphocytes, all of which are antigen-presenting cells.

$CD4^+$ T-lymphocytes respond to tumour-associated antigens presented by MHC class II molecules (Robbins and Kawakami, 1996). Dendritic cells infiltrating the tumour bed and present in the draining lymph nodes are critical for the presentation of processed tumour antigen peptides to the lymphocyte and its activation (Schuler and Steinman, 1997). Tumour cells have been generally considered to be poor stimulators of lymphocyte due to the lack of expression of HLA class II molecules and their variable expression of HLA class I molecules. Both $CD8^+$ and $CD4^+$ T-lymphocytes can destroy tumour cells by the expansion of cytoplasmic granules containing the pore-forming protein called perforin. This will result in plasma membrane disruption or by the up-regulation of Fas-ligand that can bind to Fas (on the tumours) to induce apoptosis. Cytokines are also important to tumour rejection. IFN-γ produced by both $CD4^+$ and $CD8^+$ lymphocytes is known to be critical; it leads to its activation of other effectors cells, such as macrophages, that are known to infiltrate the tumour (Brunda et al, 1995).

Tumour Antigens

Most tumours are antigenic and on the cell surfaces there are many antigens. These are divided into: tumour-specific antigens (only present in the tumour cells) others are non-specific or tumour-associated antigen (found in the embryonic and very minor quantities in the normal tissues). However, to date none of these antigens have been shown to be specific to transitional epithelium, let alone its tumours.

Bladder Tumour Antigen (BTA) in the urine has been shown to detect bladder cancer. This has been identified as human complement factor H-related protein in the urine (Habuchi et al., 2005). Malignant transformation of urothelial cells appears to be associated with loss of ABH blood group antigen and enhanced Lewis X (Le^x) antigen expression. The Le^x antigen seems to be expressed regardless of bladder cancer grade or stage (Golijanin et al., 1995).

How does immune surveillance break down?

There are many mechanisms which could explain the immune surveillance break down: impaired antigen presentation, where the recognition of tumour cells by T cells, may be impaired due to defective antigen processing and presentation (Pawelec et al, 1997, Seliger et al, 1997). Others involve the secretion of immunosuppressive products. These products may be secreted by the tumour cells themselves or by surrounding inflammatory cell infiltrates. The primary inhibitory effect of IL-10 is believed to be related to its ability to down-regulate MHC class II expression on monocytes/dendritic cells, resulting in reduced antigen presentation (Uzzo et al, 2000). IL-10 may also directly block T cell growth and may inhibit the production of other pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α.

The tumour cells secrete factors that stimulate angiogenesis such as Vascular Endothelial Growth Factor (VEGF). VEGF can induce capillary growth into the tumour, supplying required nutrients and allowing for tumour expansion. Thus, angiogenesis is a necessary and required step for transition from a small harmless cluster of cells, to a large tumour. Angiogenesis is also required for the spread of a tumour or metastasis (Walsh et al., 2002).

1.8 Inflammation and cancer

1.8.1 Introduction

Recently chronic inflammation has been identified as a risk factor for cancer. Chronic activation of the immune system is, in itself, associated with the development of tumours such as schistosomiasis and bladder cancer (Badawi et al, 1995), human papilloma virus (HPV) and cancer (Munger , 2002), helicobacter pylori bacterial infection and gastric adenocarcinoma (Naumann, 2002), the hepatitis B virus, cirrhosis and hepatocellular carcinoma (Hilleman, 2003). However, chronic inflammation, arising as a result of chronic exposure to a non-infective irritant, may also be associated with the development of malignant disease. Chronic bronchitis and emphysema due to cigarette smoking are recognised risk factors for the development of lung cancer (Mayne et al, 1999). Carcinoma of the gastro-oesophageal junction is associated with chronic oesophagitis, including Barrett's oesophagus (McCann, 1999). Mesothelioma arises as a result of chronic exposure to asbestos fibres (Wagner et al., 1960). The association between chronic inflammatory bowel disease and cancer of the bowel is well established (Kirk and Clements, 1999).

This evidence would suggest that an inflammation environment would support tumour development. It may be that the inflammatory response due to tumour environment stress may generate a protective shield from the immune system. It has been recently demonstrated that the tumour microenvironment highly resembles an inflammation site, with significant advantages for the progression of tumour, including the effect of cytokines, chemokines, leucocytes, lymphocytes and macrophages resulting in both vasodilatation and neovascularisation for increased blood flow and the immunosuppression which contributes to the process of metastasis (Schwartsburd, 2003).

1.8.2 Inflammatory cells infiltrate in cancer

T-Lymphocytes

T-lymphocytes are specific killer cells; their function is believed to be the lysis of tumour cells. There are two major types of T-lymphocytes:

- Helper T-lymphocytes (CD4+) activate macrophages, and also produce cytokines that induce the proliferation of B and T cells.
- Cytotoxic T-lymphocytes (CD8+) recognise tumour cells by using T-cell receptors to probe the surface of other cells

The receptors for T-lymphocytes are encoded by identical genes. The co-receptor for CD4+ binds with HLA class II molecules whereas the co-receptor CD8+ binds with HLA class I molecules. If a macrophage with HLA of class I presents antigen to immature T-lymphocytes, it forms clones of cytotoxic T-lymphocytes. On the other hand, if a macrophage with HLA class II molecules presents the antigen, then it forms clones of helper T-lymphocytes. Each CD8+ T-lymphocyte destroys only a limited number of tumour cells after which the store of energy and perforins in these lymphocytes becomes depleted and the lymphocytes die out on their own (Walsh et al., 2002).

Helper T-lymphocytes (CD4+) can be viewed as the main regulator by secreting cytokines; CD4+ lymphocytes influence the function of virtually all other cells of the immune system, including other T-lymphocytes, B-lymphocytes, macrophages, and natural killer cells. However, CD4+ lymphocytes are divided into two functionally distinct populations which have been recognised on the basis of the different cytokines they produce (Abbas et al, 1996). The T-helper type I (Th1) subset synthesises and secretes IL-2 and interferon-γ (IFN-γ) but not IL-4 or IL-5, whereas T-helper type II (Th2) produces IL-4, IL-5 and IL-13 but not IL-2 or IFN-γ (Kidd, 2003).

Lymphocytic infiltrations in tumour have been reliably demonstrated in a variety of solid tumours, including bladder cancer (Tanaka et al., 1970, Stavropoulos et al., 1998, Bevers et al., 2004) and suggest the presence of a local immuno-surveillance active immune defence of the host against bladder tumour (Tsujihashi et al., 1989). In addition, bladder tumours with marked lymphocytic infiltration have a better response to BCG (Alexandroff, 1999). It was Tanaka and co-workers in 1970 who noticed that there is a significant lymphocytic infiltration to the bladder tumour; they reviewed 1090 specimens of bladder tissue taken from 762 patients. They showed that lymphocytic infiltration is significantly more frequently associated with bladder cancer than benign lesions. Another interesting finding is that there was no correlation between infected urine proven on urine culture and lymphocytic infiltration (Tanaka et al., 1970). The study did not however show a correlation between tumour lymphocytic infiltration and tumour aggressiveness, but this may have been due to the relatively short follow-up of 3 years.

Natural Killer Cells (NK cells):

NK cells are natural, non-specific tumour killers and they play an important element in the anti-cancer system. The unique feature of NK cells is that they can destroy the cells in which there are low HLA class I molecule expressions. The major function of NK cells in fighting cancer is likely to be in surveillance and elimination of cells that become malignant before they can cause a tumour (Orange, Ballas, 2006). After the close contact of NK cells with cancer cells, the NK cell secretes perforins which lay out on the cancer cell surfaces to form pores and through which fluid will start to enter the cell through. The cancer cell slowly swells and at the end bursts. On the NK cell surfaces there are many stimulating molecules or activators. They are the

receptors for IFN, IL-2, IL-12, IL-15, IL-18 molecules. On the surface of all the NK cells can be found the so called the Fas-ligand molecules (CD178), which can trigger a cell death programme in the targeted cells. The activation of NK cells by interleukin 2 and interleukin 12 leads to an intensive expression of CD178 in NK cells. NK cells secrete gamma interferon which will stimulate T-helper 1 cells and inhibit T-helper 2 cells. A molecule important in down-regulating NK cell activity is prostaglandin E_2, which can be secreted by macrophages and tumour cells (Walsh et al., 2002, Nairn and Helbert, 2002).

Macrophages

Macrophages are non-specific tumour killer cells which play a major element in the tumour surveillance role by differentiating normal from neoplastic cells. Macrophages are thought to be involved in four recognised functions that affect tumour immunity: phagocytosis, modulation of immunologic activation of lymphocytes, suppression of the immune response and direct destruction of tumour cells. Once the macrophages are activated, they increase phagocytosis, increase secretion of hydrolytic enzymes, release prostaglandin E, regulate granulocyte/macrophage stem cell growth and suppress cellular proliferation. Macrophages present all types of antigens to T-lymphocytes in association with MHC class II molecules (Lewis and Pollard, 2006).

Compelling evidence has emerged in recent years that the macrophage plays an important role in tumour invasion, proliferation, angiogenesis, immunosuppression and metastasis (Lewis and Pollard, 2006). Macrophage concentration and functional state at the tumour site affect the anti-tumour activity. Phenotypes of macrophage that infiltrate a tumour are called tumour-associated macrophages (TAM). TAMs are derived from circulating monocytic precursors, and are directed into the tumour by chemo-attractant

cytokines such as colony-stimulating factor and chemokines. It is believed that the anti-tumour activity is due to the ability to secrete vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor, tumour necrosis factor alpha (TNF alpha) and IL-8 (Lewis and Pollard, 2006). High TAM numbers have been shown to be an independent prognostic factor in many tumours including bladder cancer (Hanada et al., 2000, Bingle et al., 2002). Hanada and co-workers examined the relation between TAM, microvessel density and clinical outcome in 63 bladder cancer patients with a mean follow-up of 65 months. They found that TAM was higher in invasive bladder cancer and have a significant correlation with microvessel density. In addition, they found on multivariate analysis TAM and MCV were independent prognostic parameters (Hanada et al., 2000).

Dendritic cells

Dendritic cells are leucocytes that are highly specialised in the capture and presentation of antigens to T-lymphocytes. They can process the antigens, become activated, mature and migrate into lymphoid tissue, where they present antigen to lymphocytes to produce a specific immune reaction. Dendritic cells differ from macrophage and monocyte antigen-presenting cells because they are potent initiators of primary naive T-lymphocyte responses. They are believed to control the induction (and, possibly, the suppression) of antigen-specific immune responses (Nencioni and Brossart, 2004).

Injection of dendritic cells loaded with tumour-associated antigens into patients was shown to break tolerance and to induce anti-tumour cytotoxic immune responses. The dendritic cells- based clinical trials performed so far have demonstrated that this form of immunotherapy is feasible and safe (Banchereau et al., 2001).

In summary, T-lymphocytes can destroy tumour cells directly by apoptosis or perforin-mediated killing and indirectly by macrophages. NK cells are involved in immune surveillance and mediate tumour cell killing by the lytic protein perforin. Macrophages are principal elements in immune surveillance. They release molecules that kill or inhibit tumour cells. B cells are indirectly involved in tumour cell killing through the formation of the antigen-antibody complex. Both cell-mediated and humoral immunity have been demonstrated to have anti-tumour activity.

1.8.3. Cytokines

Cytokines are soluble protein substances produced by a wide variety of cell types and are critical to the functioning of both innate and adaptive immune responses. Cytokines have been variously named as lymphokines, interleukins and chemokines, based on their presumed function, and their cell of secretion or target of action.

Cytokines can be divided into two groups according to their function in relation to inflammation: promoting inflammation and are called pro-inflammatory cytokines; whereas other cytokines suppress inflammation and are called anti-inflammatory cytokines (Cytokines Web, 2006). However, there is no clear division between the two, e.g. IL-4, IL-10, and IL-13 are potent activators of B lymphocytes. On the other hand, IL-4, IL-10, and IL-13 are also potent anti-inflammatory agents. Another classification divides cytokines into those that promote the proliferation and functioning of helper T-cells type 1 (IL-1, IL-2, IL-3, IL-12 and INF-γ) and helper T-cells type 2 (IL-4, IL-5, IL-6, IL-10, IL-13 and TGF-β) (Figure 1.5).

In bladder cancer, high T-helper-type 1 (Th1) cytokines (INF γ, IL-12 and IL-2), after BCG treatment has been observed to be a common feature in BCG responders. In contrast, higher levels of Th2 cytokines IL-10 and/or IL-6 appear to be associated with BCG failure (O'Donnell et al., 1996).

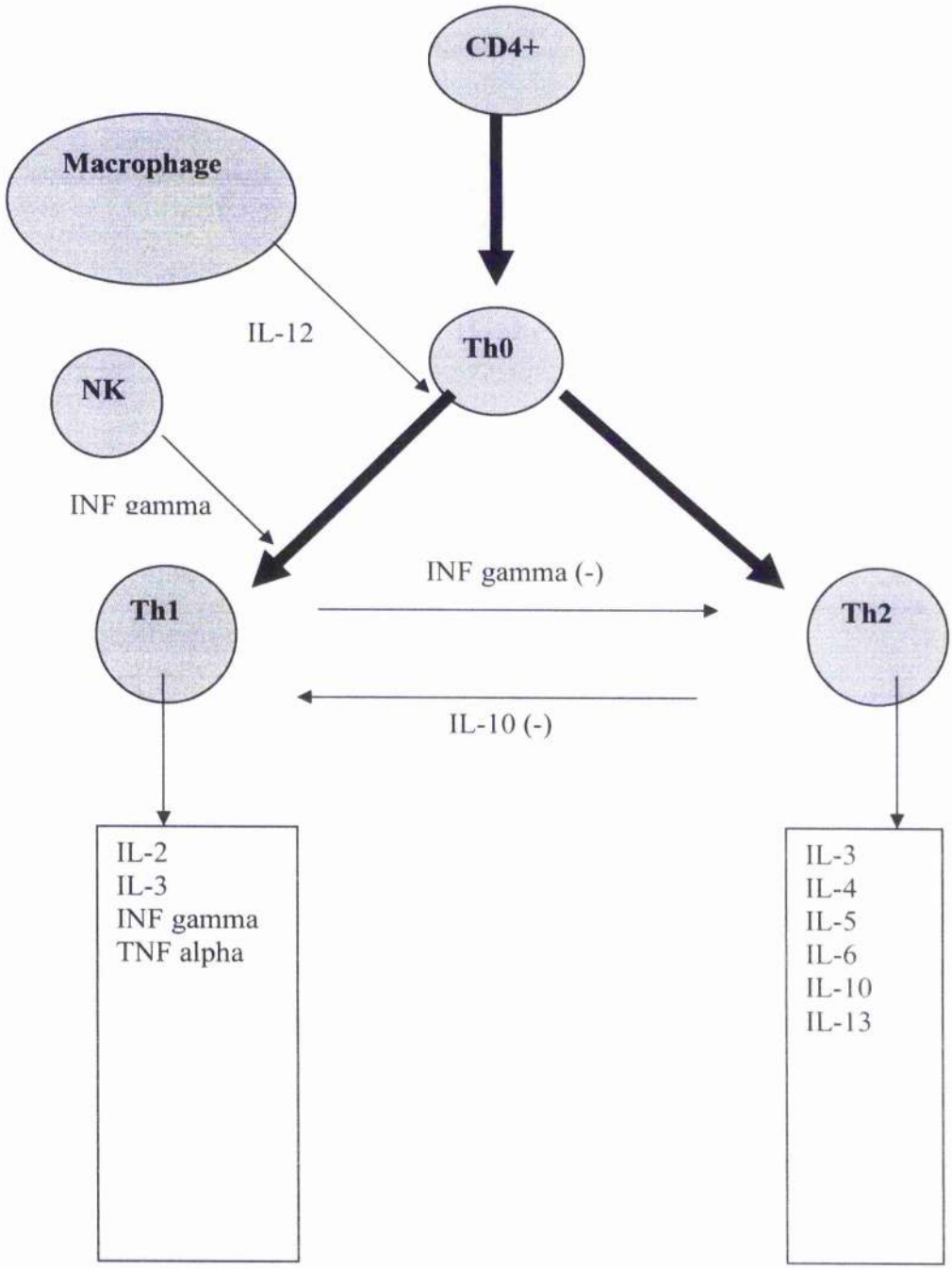

Figure 1.5 The production of cytokines.

Interleukin-1 (IL-1) takes part in nearly all the phases of an immune response. IL-1 appears to activate cytotoxic T-lymphocytes and NK cells and takes part in the regulation of IL-2, IL-4, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF). The active inhibitors of IL-1 production are IL-4, IL-10, IL-12 and the tumour necrosis factor –alpha (Kornman, 2006).

There are two types of this interleukin: IL-1α and IL-1β which differ from each other in their bioactivities. IL-1 α, for example, appears to strengthen the weakening anti-tumour immunity, slows down tumour growth and lowers its metastatic potential. IL-1β, on the other hand, appears to reinforce the regeneration of tissue and stimulates the metastatic processes (Kornman, 2006).

For example, Seddighzadeh and co-workers (2003) reported that in 167 bladder tumours with a median follow-up of 56 months, low levels of IL-1α expression were associated with an increased risk for cancer-specific death.

Interleukin-2 also appears to increase the function of T-cytotoxic lymphocytes and NK cells, promotes the production of perforins and IFN by these cells, activates the monocytes and macrophages which help to synthesise and secrete TNF, IL-1, IL-6, IL-8, and granulocyte-colony stimulating factor (G-CSF). The function of IL-2 is dependent on its receptors IL-2 (IL-2R) (Nelson, 2004). It has been shown that the inhibition of IL-2 is associated with the accumulation of the immuno-suppressive substances, mainly the prostaglandins, immune complexes and the metabolic products of cancer cells developing an immunosuppression state (Nelson, 2004).

Interleukin-6 plays an important role in the anti-tumour activity in a number of ways. It is a pro-inflammatory cytokine; it modulates the anti-tumour activities of macrophages and stimulates the production of C-reactive protein. Interleukin-6 regulates the immune system via the proliferation and activation of cytotoxic T cells,

proliferation and differentiation of B cells, and production of acute phase proteins. In addition, IL-6 has been shown to regulate cell growth, differentiation and survival in other target cells (Horn et al., 2000).

Increased local IL-6 expression has been demonstrated in various cancers including bladder cancer. In addition, IL-6 takes part in pathogenesis of anorexia, cachexia and anaemia in cancer patients (Andrews et al., 2002). By blocking the genes or introducing the antibodies against IL-6, these clinical complications of cancer e.g. anorexia, cachexia and anaemia, reduce to milder forms. Andrews and co workers found that plasma IL-6 levels were higher in patients with bladder cancer than in healthy controls. Levels of IL-6 and IL-6sR were associated with cancer stage and metastases, and were strong independent predictors of disease recurrence and disease-specific survival (Andrews et al., 2002).

Interleukin-10 produced by T-lymphocytes, monocytes and macrophages, has a wide range of anti-inflammatory effects. The anti-inflammatory effect of IL-10 is expressed in terms of its ability to lower the production of pro-inflammatory cytokines, increase the production of IL-1 antagonist receptors and decrease the adhesion of leucocytes onto endothelial cells activated by IL-1. An elevated IL-10 level is associated with a raised IL-12 production and a reduction in IFN production (Mocellin et al., 2005). A high concentration of IL-10 is also a bad prognostic sign and generally corresponds with a rapid tumour growth and favours tumour escape from immune surveillance. However, a wealth of evidence is accumulating that IL-10 also possesses some immuno-stimulating properties. In fact, IL-10 has the combined ability of influencing positively and negatively the function of innate and adaptive immunity (Mocellin et al., 2005).

Interleukin-12 is an activator of cellular immunity with anti-tumour and anti-metastatic activities. IL-12 activates the cytotoxicity of macrophages; whereas the deficiency of its production by macrophages can significantly reduce the anti-tumour activities. IL-12 suppresses the development of cachexia and anaemia induced by IL-1 and IL-6 in cancer patients. IL-12 stimulates the differentiation of T-lymphocytes to a T-helper 1 phenotype which in turn produce high amounts of IFN-γ and cytotoxic cells. These biological properties seem useful in immune therapy for bladder cancer (Clinton et al., 2000).

Tumour Necrosis Factor TNF (cachexin) is an important cytokine involved in systemic inflammation and the acute phase response. TNF is released by leucocyte, endothelium and several other tissues in the course of damage. Its release is stimulated by several other mediators, such as IL-1. Some pre-clinical findings suggest that TNF may promote cancer development and progression, which has led to the proposition of anti-TNF therapy as a novel approach to malignancies (Mocellin et al., 2005). TNF has a number of actions on various organ systems generally, together with IL-1 and Il-6:

- On the hypothalamus: stimulating of the hypothalamic-pituitary-adrenal axis by stimulating the release of corticotrophin-releasing hormone (CRH), suppressing appetite (hence its name "cachexin") and fever.
- On the liver: stimulating the acute phase response, leading to an increase in C-reactive protein and a number of other mediators.
- It attracts neutrophils very potently, and helps them to stick to the endothelial cells for migration.
- On macrophages: stimulating phagocytosis, and production of IL-1 and prostaglandin E2.

Interferons INF are natural proteins produced by the cells of the immune systems and are divided into interferon-alpha, beta and gamma. Interferon-alpha and beta are produced by many cell types, including T-lymphocyte and B-lymphocyte, macrophages, fibroblasts, endothelial cells, osteoblasts and others. They stimulate both macrophages and NK cells. Interferon-gamma has anti-viral and anti-tumour effects; stimulates macrophages and recruits leucocytes, resulting in increased inflammation. Interferon-gamma strengthens the effects of interferon-alpha and interferon-beta (Gattinoni et al., 2006)

Granulocyte Colony-Stimulating Factor G-CSF stimulates the survival, proliferation, differentiation, and function of neutrophils, granulocyte precursor cells and mature neutrophils. G-CSF is produced by endothelium, macrophages, and a number of other immune cells. The G-CSF-receptor is present on cells in the bone marrow and in response to stimulation by G-CSF, initiates proliferation and differentiation into mature granulocytes (Lopez-Lazaro, 2006).

1.8.4 Acute-phase proteins

Acute-phase proteins are plasma proteins, mostly synthesised in the liver, and their plasma concentrations may increase several hundred-fold as part of the response to inflammatory stimuli. They promote inflammation, activate the complement cascade, and stimulate phagocytes (Du Clos, 2000). Three of the best-known examples of these proteins are C-reactive protein, fibrinogen, and serum amyloid A protein (figure 1.6). However, C-reactive protein is widely used because its measurement is standardised in the laboratory and available in hospitals. These proteins are synthesised in the liver by the hepatocytes and their synthesis is regulated by cytokines; especially IL-6, this synthesis is enhanced synergistically by IL-1β where IL-1β enhances IL-6 induction through NF-κB (for C-reactive protein and fibrinogen) and IL-1 and TNF (for serum amyloid A) (Gabay and Kushner, 1999).

C-reactive protein was originally discovered by Tillett and Francis in 1930 as a substance in the serum of patients with acute inflammation that reacted with the C polysaccharide of pneumococcus. The C-reactive protein gene is located on the first chromosome. The function of C-reactive protein is believed to activate complement, binds to Fc receptors and acts as an opsonin for various pathogens. Interaction of C-reactive protein with Fc receptors leads to the generation of pro-inflammatory cytokines that enhance the inflammatory response (Du Clos, 2000). C-reactive protein recognises altered self and foreign molecules based on pattern recognition. Thus, C-reactive protein is thought to act as a surveillance molecule for altered self and certain pathogens. This recognition provides early defence and leads to a pro-inflammatory signal and activation of the humoral, adaptive immune system (Marnell et al., 2005). High acute phase proteins were demonstrated in bladder cancer (Bastable et al., 1979, O'Quigley et al., 1981).

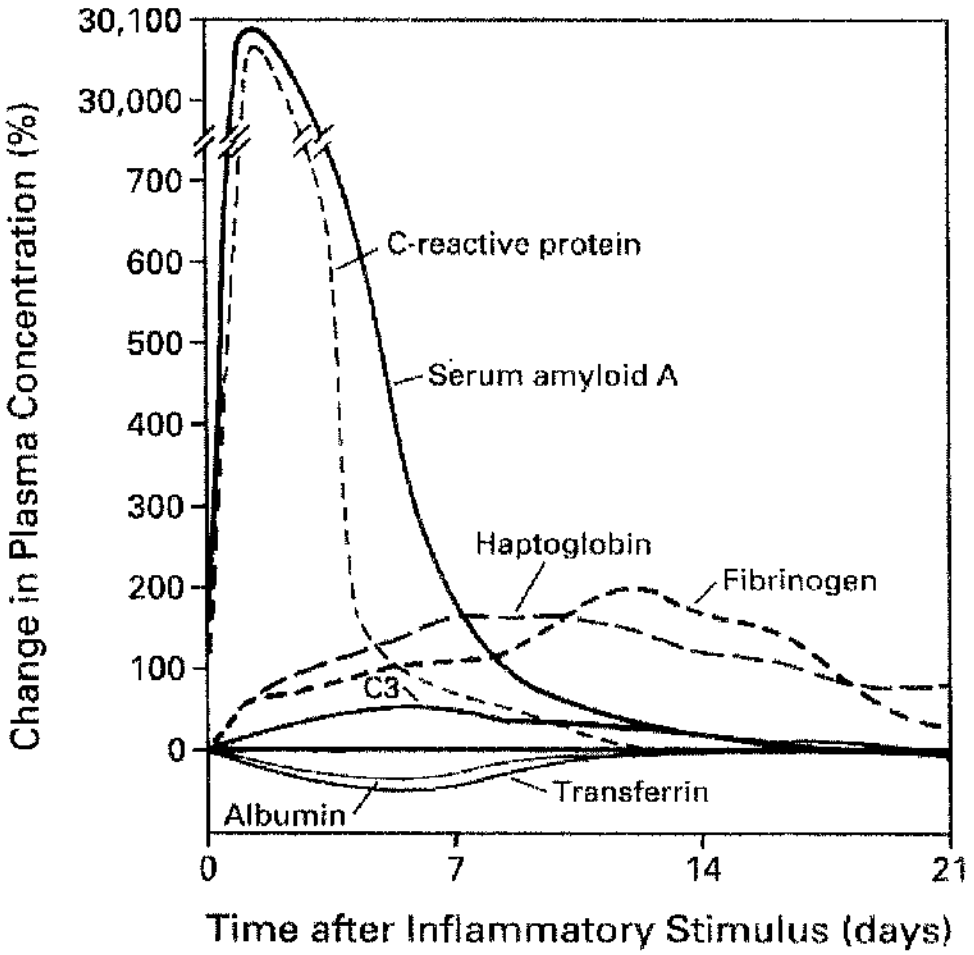

Figure 1.6 Changing pattern of acute phase proteins after a moderate inflammatory stimulus (Adapted from Gabay and Kushner, 1999).

In summary

Acute inflammation starts a cascade of cytokines and chemokines that attract immune and non-immune cells, mainly neutrophils, to infiltrate disrupted and damaged tissue. The process of acute inflammation is usually self-limiting because the production of pro-inflammatory cytokines gives way to anti-inflammatory cytokines as healing progresses. On the other hand, chronic inflammation is considered to be inflammation of prolonged duration in which active inflammation, tissue destruction, and attempts at repair are proceeding simultaneously. Although it may follow acute inflammation, chronic inflammation frequently begins insidiously, as low-grade, in this case, mononuclear cells, macrophages, lymphocytes, and plasma cells are found in addition to neutrophils, and active tissue destruction and repair proceed simultaneously (Figure 1.7).

It has been hypothesised that the origin of many cancer is at sites of chronic inflammation (Virchow 1863). Although it is now clear that proliferation of cells alone does not cause cancer, sustained cell proliferation in an environment rich in inflammatory cells, growth factors, activated stroma, and DNA-damage-promoting agents, appears to promote neoplastic risk (Balkwill and Mantovani, 2001).

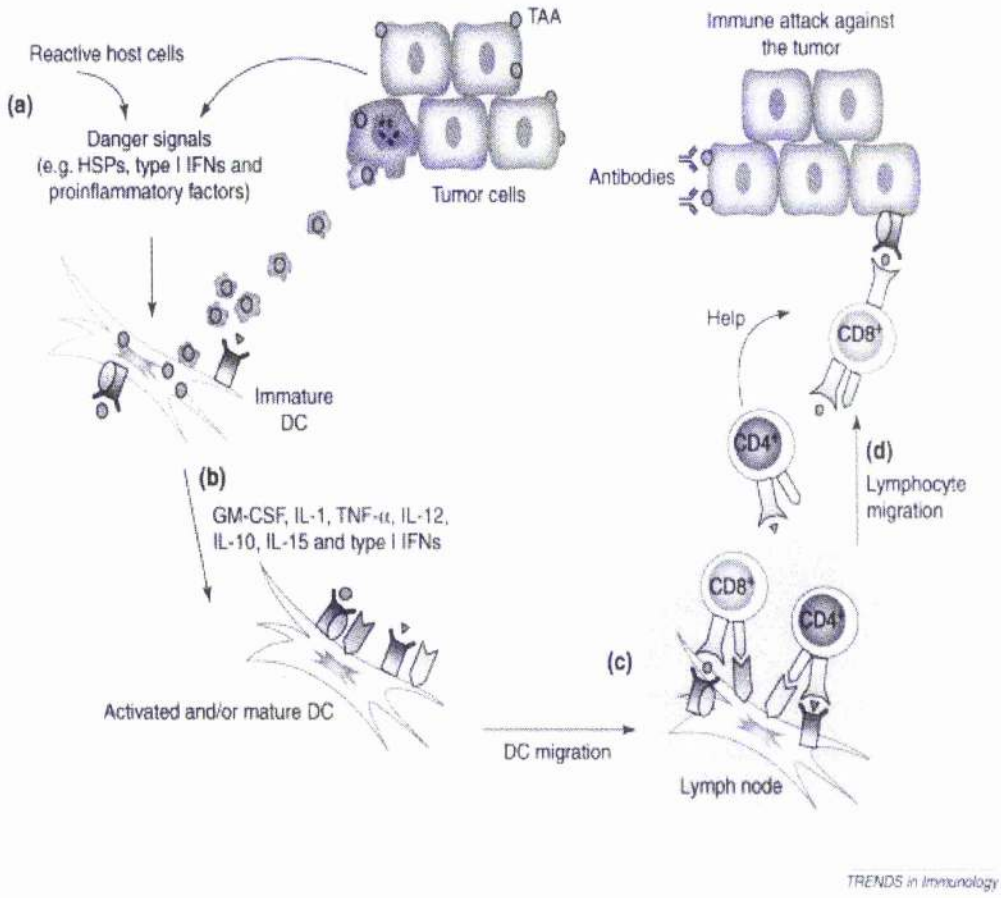

Figure 1.7 The events which occur in the course of an immune response against tumour cells (adapted from Belardelli and Ferrantini, 2002).

2. HYPOTHESIS AND AIMS

The inter-relationship between tumour and host factors and disease progression in patients with transitional carcinoma of the urinary bladder is not clear. From the previous text, it would appear that the host inflammatory response plays an important role in the progression of bladder cancer. The presence of the tumour cells appears to activate the innate immune system as part of the host reaction to the presence of tumour. One scheme might be that the attraction and activation of various inflammatory cells at or near the tumour site leads to the release of various cytokines of which some will act locally on other inflammatory cells while others will find their way into the circulation and activate the systemic inflammatory response. The release of cytokines such as IL-6 into the circulation will activate the release of the acute-phase proteins in the liver. The adaptive immune system will be activated due to the presence of antigens in the tumour. These tumour specific antigens will activate the humoral immune system and add to the inflammatory response. Therefore, rising acute phase proteins are usually a sign of disturbance of the host-tumour relationship.

The hypothesis of the present investigations is that there are significant local and systemic inflammatory responses and that these play a role in determining cancer-specific survival of patients with transitional cell carcinoma of the urinary bladder.

The aims of the thesis were to examine:

1. The prognostic value of the systemic inflammatory response in patients with transitional cell carcinoma of the urinary bladder.

2. The relationship between the systemic inflammatory response, tumour proliferative activity, T-lymphocytic infiltration, COX-2 expression, tumour associated macrophage and microvessel density and survival in patients with transitional cell carcinoma of the urinary bladder

3. The relationship between the systemic inflammatory response and the cytokines/ chemokines profile in patients with transitional cell carcinoma of the urinary bladder.

CHAPTER 3: THE RELATIONSHIP BETWEEN THE SYSTEMIC INFLAMMATORY RESPONSE AND SURVIVAL IN PATIENTS WITH TRANSITIONAL CELL CARCINOMA OF THE URINARY BLADDER.

3.1 Introduction

Bladder cancer is the fourth most common malignancy in the United Kingdom in men and the eighth in women, with a total of 12,500 new cases each year causing 5,000 deaths annually (CancerStats, 2002). More than 90% of bladder cancers diagnosed in the Western world are transitional cell carcinomas and constitute a significant proportion of the general urological consultant's workload due to its high prevalence and recurrent nature.

It is increasingly recognised that, in addition to tumour stage, disease progression is dependent on a complex interaction of the tumour and host inflammatory response (Balkwill and Mantovani, 2001; Coussens and Werb, 2003). Indeed, the systemic inflammatory response, as evidenced by elevated circulating concentrations of C-reactive protein, has been shown to be a stage-independent prognostic factor in a variety of operable tumours (Mahmoud and Rivera, 2002; McMillan et al., 2003; Ikeda et al., 2003). To our knowledge there has been only one previous study which has examined the prognostic value of C-reactive protein in patients with invasive bladder cancer (O'Quigley et al., 1981).

The aim of the present study was to examine the relationship between stage, grade, C-reactive protein and cancer-specific survival in patients with superficial or invasive bladder cancer.

3.2 Patients and methods

Patients with biopsy-proven transitional cell carcinoma presenting to a single urological unit at Glasgow Royal Infirmary between 1992-1999 were reviewed. A cohort of 105 patients were selected on the basis of having a measurement of C-reactive protein prior to or 3–6 months following transurethral resection of bladder tumour. These were part of a larger cohort of 457 patients who underwent transurethral resection between 1992 and 1999 in Glasgow Royal Infirmary. The selected patients were similar in terms of age, sex, stage and grade compared with the original cohort (Table 3.1).

Those patients with a measurement of C-reactive protein prior to or 3-6 months following elective admission for transurethral resection of bladder tumour were included in the study as previously described (McMillan et al., 2003). Tumour stage was assessed using the UICC guidelines, and tumour grade performed according to the WHO grading system. Date and cause of death were obtained from clinical follow-up notes and corroborated with that from the Cancer Registry, Scotland using ICD codes (bladder cancer 1889).

Routine laboratory measurement of patients' serum for C-reactive protein concentration was performed. The limit of detection of the assay was a C-reactive protein concentration lower than 5 mg/l. The coefficient of variation, over the range of measurement, was less than 5% as established by routine quality control procedures. C-reactive protein measurement of greater than 10 mg/l was considered to indicate the presence of systemic inflammatory response. (O'Gorman et al., 2000).

The Research Ethics Committee of North Glasgow NHS Trust approved the study.

3.3 Statistical analysis

Survival analysis was performed using the Cox proportional hazard model with patient age, sex, tumour stage and grade and C-reactive protein concentration as prognostic variables. Deaths up to September 2004 were included in the analysis.

Multivariate survival analysis was performed using stepwise backward procedure to derive a final model of the variables that had a significant independent relationship with survival. To remove a variable from the model, the corresponding P value had to be greater than 0.10. Analysis was performed using SPSS software (SPSS, Chicago, Illinois, USA).

3.4 Results

The characteristics of patients with bladder cancer (n= 105) are shown in Table 3.2). The majority were male, over the age of 65 years, had superficial disease (Ta, T1) and had elevated C-reactive protein concentrations either pre- or post-operatively. Twenty four patients had adjuvant therapy (8 radiotherapy, 8 BCG, 8 cystectomy).

Thirty two patients had C-reactive protein concentrations determined both prior to and following the resection. Of the 20 patients with an elevated pre-operative value, 12 (60%) had a raised concentration after the operation. Of the 12 patients with a normal pre-operative value, 9 (75%) had a post-operative value in the normal range. Raised C-reactive protein concentrations was associated with high tumour grade (Figure 3.1)

During the follow-up period 80 patients died, 41 of them from bladder cancer and the mean cancer specific survival was 82.9 months (95% Confidence Interval 70.8-94.9). On univariate analysis, stage ($p<0.001$), grade ($p<0.01$) and elevated C-reactive protein pre-operatively ($p<0.05$) and post-operatively ($p<0.01$) were significantly associated with overall survival. Also, stage ($p<0.001$), grade ($p<0.001$) and elevated C-reactive protein pre-operatively ($p<0.05$) and post-operatively ($p<0.01$) were significantly associated with cancer-specific survival (Table 3.3).

On multivariate analysis of patients who had a pre-operative C-reactive protein determination, stage (HR 4.00, 95%CI 2.02-7.94, $p<0.001$) and pre-operative C-reactive protein (HR 2.73, 95%CI 1.23-6.07, $p=0.014$) were independently associated with overall survival. Also, stage (HR 3.37, 95%CI 1.37-8.29, $p=0.008$), grade (HR 2.01, 95%CI 1.14-3.57, $p=0.017$) and pre-operative C-reactive protein (HR 3.31, 95%CI 1.09-10.09, $p=0.035$) were independently associated with cancer-specific survival. Those patients with an elevated pre-operative C-reactive protein concentration had a

mean cancer-specific survival of 65.5 months (95%CI 46.8-84.2) compared with 103.7 months (95%CI 81.8-125.6) in those patients with a C-reactive protein concentration in the normal range (Figure 3.2).

3.5 Discussion

In the present study, both tumour grade and the presence of a systemic inflammatory response, as evidenced by elevated circulating concentrations of C-reactive protein, were independent predictors of cancer-specific survival in patients with bladder cancer. These results confirm the importance of tumour grade as a stage-independent prognostic factor (Ali-El-Dein et al, 2003; Nishiyama et al, 2004) and are consistent with the work of O'Quigley et al (1981), who reported that an elevated C-reactive protein concentration, measured by radial immunodiffusion and using the limit of sensitivity of 12mg/l as a cut-off, was associated with poor survival in patients with invasive bladder cancer.

Furthermore, because C-reactive protein concentration is independent of tumour stage and grade, the presence or absence of a systemic inflammatory response might form the basis of a new prognostic score that reflects not only the tumour but also the host response. Indeed, this approach has recently been used in both lung and colorectal cancer (Forrest et al., 2003; Canna et al., 2004).

There has been only one previous report on which to base a sample size. O'Quigley and co-workers (1981) reported a significant relationship between elevated C-reactive protein concentrations and survival in 82 patients with invasive bladder cancer. The numbers of patients who died in the follow-up period was not stated. In the present study we had 105 patients, superficial and invasive disease, of whom 80 (76%) patients died during the follow-up period. Therefore, it is likely that the results of the present study are reliable and are consistent with the only other previous published study.

Although in the present study, it may appear from the present results that an elevated post-operative C-reactive protein concentration is a better predictor of cancer-specific survival than an elevated concentration prior to resection, in a clinical context the measurement of pre-operative CRP concentration is likely to be of more value because it would allow planning of adjuvant therapy.

The mechanisms by which a systemic inflammatory response might impact on cancer-specific survival are not clear. However, it is known that as part of the systemic inflammatory response to the tumour there is a release of pro-inflammatory cytokines and growth factors which not only stimulate tumour growth (Abramovitch et al., 1999; Coussens and Werb, 2003) but also produce profound catabolic effects on host metabolism (Kotler, 2000). For example, interleukin-6, produced by the tumour or infiltrating inflammatory cells, is recognised as a growth promoter in bladder cancer (Okamoto et al., 1997; Andrews et 2002). Interleukin-6 also stimulates liver production of acute-phase proteins, such as C-reactive protein, which increases the demand for certain amino acids, which, if limited in the diet, must be obtained from the breakdown of skeletal muscle (Preston et al., 1998; McMillan et al., 1998). In this way, the presence and magnitude of a chronic systemic inflammatory response may produce a progressive nutritional and functional decline, ultimately resulting in reduced survival.

In summary, it would appear that the systemic inflammatory response is a stage and grade-independent prognostic factor in patients with bladder cancer. The role of the systemic inflammatory response in determining disease-specific survival in patients with bladder cancer is worthy of further study to establish its value as a prognostic factor.

Table 3.1 Clinical characteristics for all patients with bladder cancer attended between (1992-1999) and the selected cohort.

		Original cohort	Selected cohort
		N=457 (100%)	n=105 (100%)
Age	≤65 yrs	146 (32)	37 (35)
	>65 yrs	220 (68)	68 (65)
Sex:	Male	329 (72)	75 (71)
	Female	128 (28)	30 (29)
Stage	Superficial (Ta, T1)	352 (77)	76 (72)
	Invasive	105 (23)	29 (28)
Grade	G1	169 (37)	36 (34)
	G2	151 (33)	31 (30)
	G3	137 (30)	38 (36)

Table 3.2 Clinical characteristics and survival in patients with bladder cancer.

		Patients
		105 (100%)
Age	≤65 yrs	37 (35)
	>65 yrs	68 (65)
Sex:	Male	75 (71)
	Female	30 (29)
Stage	Superficial (Ta,T1)	76 (72)
	Invasive	29 (28)
Grade	G1	36 (34)
	G2	31 (30)
	G3	38 (36)
Pre-operative		
C-reactive protein	≤10 mg/l	16 (15)
	>10 mg/l	43 (41)
Post-operative		
C-reactive protein	≤10 mg/l	36 (34)
	>10 mg/l	42 (40)
Adjuvant therapy		
No		81 (77)
Yes		24 (23)
Alive		25 (24)
Dead		
Bladder cancer-specific		41 (39)
Intercurrent		39 (37)

Table 3.3 The relationship between variables and overall and cancer-specific survival in patients with bladder cancer: univariate analysis

	Overall		Cancer-specific	
	Hazard Ratio	P-value*	Hazard Ratio	P-value*
Age (≤65/ >65yrs)		0.063		0.142
Sex (Male/ Female)		0.338		0.803
Stage				
(Superficial/ Invasive)	2.82 (1.74-4.57)	<0.001	4.96 (2.61-9.41)	<0.001
Grade (G1/ G2/ G3)	1.48 (1.14-1.92)	0.004	2.17 (1.45-3.23)	<0.001
Pre-operative				
C-reactive protein				
(≤10/ >10 mg/l)	2.50 (1.15-5.43)	0.021	3.03 (1.04-8.87)	0.043
Post-operative				
C-reactive protein				
(≤10/ >10 mg/l)	2.31 (1.34-3.97)	0.003	3.28 (1.41-7.63)	0.006
Adjuvant therapy				
(No/ Yes		0.537		0.732

Values in parentheses are 95% confidence intervals. *Cox proportional hazard model.

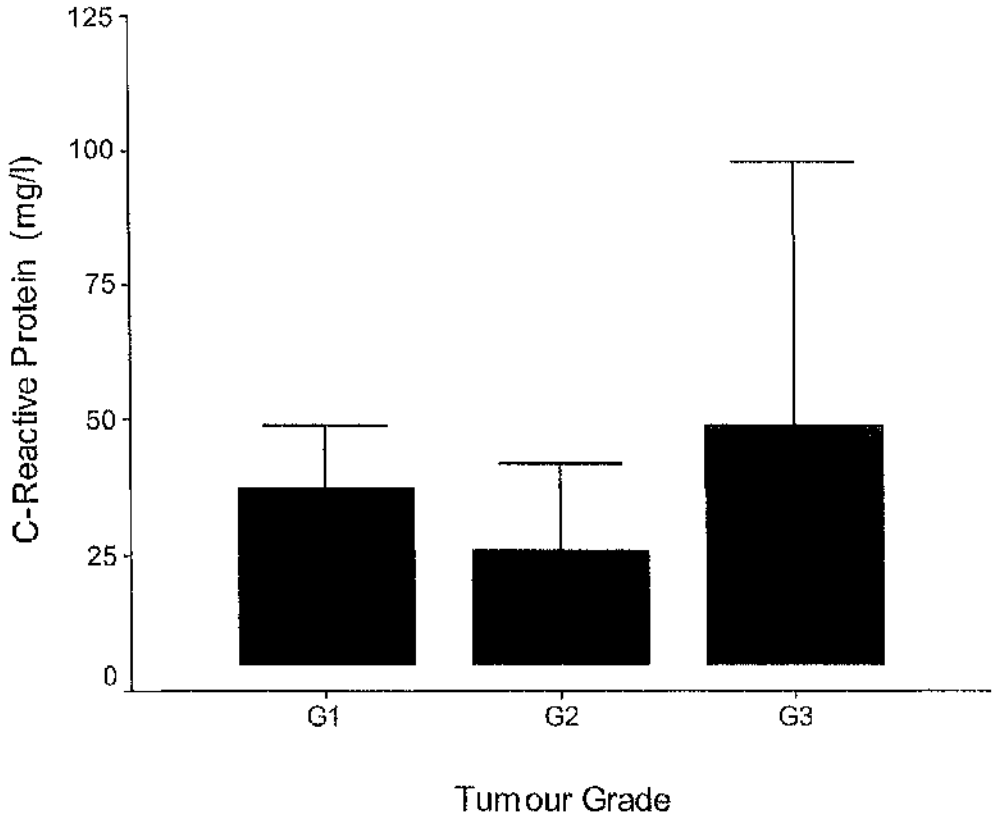

Figure 3.1 The relationship between the variance in C-reactive protein concentrations and tumour grade in patients with transitional cell carcinoma of the urinary bladder.

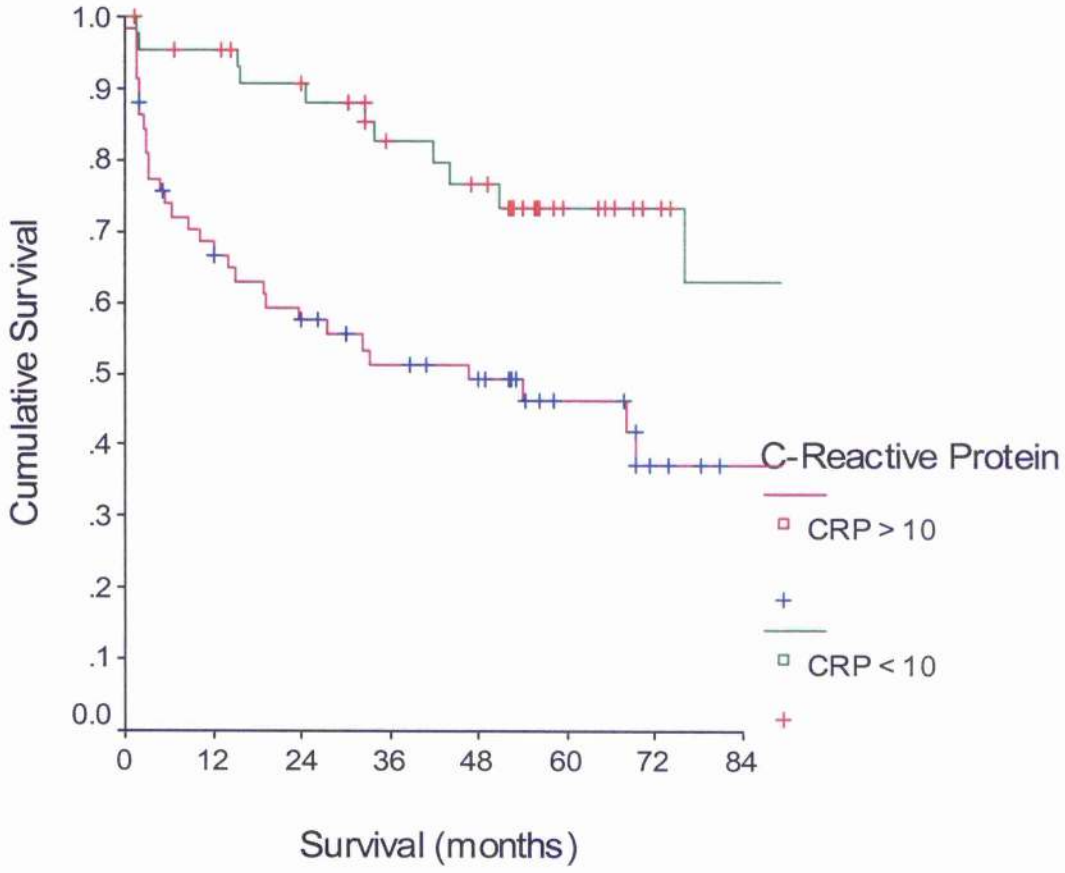

Figure 3.2 The relationship between high vs. normal C-reactive protein and survival in patients with transitional cell carcinoma of the urinary bladder.

CHAPTER 4: THE RELATIONSHIP BETWEEN THE SYSTEMIC INFLAMMATORY RESPONSE, TUMOUR PROLIFERATIVE ACTIVITY, T-LYMPHOCYTIC INFILTRATION, TUMOUR ASSOCIATED MACROPHAGE, MICROVESSEL DENSITY AND COX-2 EXPRESSION AND SURVIVAL IN PATIENTS WITH TRANSITIONAL CELL CARCINOMA OF THE URINARY BLADDER.

4.1 Introduction

It is increasingly recognised that disease progression is dependent on a complex interaction of the tumour and the host inflammatory response (Balkwill and Mantovani, 2001, Coussens and Werb, 2002, Vakkila and Lotze, 2004). Recently, the systemic inflammatory response, as evidenced by elevated circulating concentrations of C-reactive protein, has been shown to be independently associated with poorer survival in patients with advanced cancer (O'Gorman et al., 2000, Forrest el al., 2003, Maltoni et al., 2005). There is also evidence that C-reactive protein has independent prognostic value in primary operable cancer (McMillan et al., 2003, Ikeda et al, 2003, Jamieson et al., 2005, Lamb et al., 2006; Crumley et al., 2006). Therefore, it would appear that the systemic inflammatory response is of considerable importance in the relationship between the tumour, the host and outcome in patients with cancer. Recently, we have reported that an elevated C-reactive protein was associated with poor cancer-specific survival in patients with bladder cancer independent of tumour stage and grade (Hilmy et al., 2005).

The basis of the independent relationship between an elevated C-reactive protein concentration and poor survival in cancer is not clear. There are a number of possible explanations. Firstly, that an elevated C-reactive protein identifies tumours capable of

producing significant amounts of pro-inflammatory cytokines, either directly or indirectly, in particular interleukin-6 (Kinoshita et al., 1999, McKeown et al., 2004) and therefore with the potential for more rapid growth of tumour cells (Jee et al., 2001, Trikha et al., 2003) or with impaired T-lymphocytic function (Maccio et al., 1998, Canna et al., 2005) allowing unrestrained tumour growth and dissemination.

Precise localisation of pro-inflammatory cytokines to tumour cells or inflammatory cells within the tumour, particularly in paraffin embedded tissues, remains problematical (Canna et al, 2005). However, tumour proliferative activity has been reliably assessed using the Ki-67 labelling index in a variety of solid tumours, including bladder cancer (Habuchi et al., 2005, Blanchet et al., 2001). Also, infiltration of tumours with T- lymphocytes and macrophages has been reliably demonstrated in a variety of solid tumours, including bladder cancer, and appears to have independent prognostic value (Bevers et al., 2004, Stavropoulos et al., 1998; Hanada et al., 2000, Bingle et al., 2002). In addition, macrophage tumour infiltration has been shown to be higher in invasive bladder cancer and have a significant association with microvessel density and survival (Hanada et al., 2000).

Central to the local inflammatory response is cyclooxygenase-2 and increased expression has been shown to be associated with poor survival in a number of common solid tumours (Dannenberg et al., 2001, Dannenberg and Subbaramaiah, 2003). The aim of the present study was therefore to examine the relationship between the systemic inflammatory response (C-reactive protein), tumour proliferative activity (Ki-67), tumour T-lymphocyte (CD4+, CD8+) infiltration, tumour macrophage (CD68) infiltration, tumour microvessel density (CD34), tumour COX-2 expression and cancer-specific survival in patients with transitional cell carcinoma of the bladder.

4.2 Patients and Methods

A cross-sectional study of patients with biopsy-proven transitional cell carcinoma and with a measurement of C-reactive protein prior to transurethral resection of bladder tumour in Glasgow Royal Infirmary between 1992 and 2001 was carried out. Tumours were grouped according to whether they had superficial (pTa, pT1 and CIS) or muscle-invasive (pT2-pT4) disease. However, patients with G3 pT1 were considered as muscle-invasive tumours since they are recognised to have a significantly higher progression rate (Manoharan and Soloway, 2005). At this time no patient showed clinical evidence of infection, or other inflammatory conditions. Tumour stage was assessed using the 1997 AJCC/UICC TNM classification (Sobin and Witteking, 2002) and tumour grade performed according to the 1999 WHO grading system (Busch and Algaba, 2002).

The Research Ethics Committee of North Glasgow NHS Trust approved the study.

C-reactive protein measurement

Routine laboratory measurement of patients' serum for C-reactive protein concentration was performed. The limit of detection of the assay was a C-reactive protein concentration lower than 5 mg/l. The coefficient of variation, over the range of measurement, was less than 5% as established by routine quality control procedures. C-reactive protein measurement of greater than 10mg/l was considered to indicate the presence of systemic inflammatory response (O'Gorman et al., 2000).

Immunohistochemistry

Blocks from the primary tumour were fixed in 10% buffered formalin and embedded in paraffin wax. One representative block of tumour was selected for each patient. Sections (4mm) were cut and mounted on slides coated with aminopropyltriethoxysilane and placed in oven at 56 °C for 40 minutes. Slides were dewaxed in xylene for 4 minutes twice and rehydrated through graded alcohols then rinsed with water.

The following monoclonal antibodies were used: Ki-67 (Dako, Cambridgeshire, UK) at 1:200 dilution, CD4 (Vector, Peterborough, UK) at 1:10, CD8 (Dako, Cambridgeshire, UK) at 1:200, COX-2 human IgG (Cayman Chemical CO., MI, USA) at 1:800, CD68 (Dako, Cambridgeshire, UK) at 1:200 and CD34 (Novocastra, Newcastle upon Tyne, UK) at 1:100.

Antigen retrieval for Ki-67, CD4, CD8, CD68, CD34 and COX -2 was carried out by microwaving in 1mM Tris EDTA buffer (0.37g Sodium EDTA, 0.55g Tris in 1 litre distilled water) for 5 minutes under full pressure in a plastic pressure cooker in a 850W microwave on full power.

Staining method for Ki-67, CD4, CD8, CD34 and CD68 was as follows:

1. 5% Normal Goat Serum 20 minutes.
2. Primary antibody 30 minutes.
3. Wash in Tris Buffered Saline (TBS).
4. Hydrogen Peroxide blocking agent (Dako S2023)
5. Wash in TBS
6. Envision (Dako, Cambridgeshire, UK) 30 minutes
7. Wash in TBS
8. DAB (Dako, Cambridgeshire, UK) 10 minutes

COX-2 sections were immunostained using the biotinylated/streptavidin peroxidase complex (Dako, Cambridgeshire, UK) technique. Sections were then incubated in 3'3'diaminobenzidine (DAB, Dako, Cambridgeshire, UK) as a substrate for 10 minutes at room temperature.

Negative controls were stained using antibody-diluting fluid alone. Finally, copper enhancement was carried out for 5 minutes. Then, tissues were counter-stained with haematoxylin for 1 minute then washed with water, followed by scotts water for another minute before washing them with water. Slides were dehydrated through graded alcohols 70%, 90% and 100%, two minutes each, and xylene for two five minutes. The slides were cleared, mounted with Pertex and appropriate slide covers applied.

Morphometry

Ki-67

Quantitative analysis of the Ki-67 expression tumour cells was performed by identifying the Ki-67 labelling index. The percentages of Ki-67-reactive tumour cells were evaluated x 400 magnification by scoring a minimum of 1000 tumour cells in randomly selected fields (Figure 4.1). Only fields containing tumour tissues were counted. Any normal tissue on the slide was excluded from the analysis.

CD4+ and CD8+

Quantitative analysis of the lymphoid infiltrate was performed using point counting (Anderson et al., 1965) with a random sampling technique. With this method, the volume occupied by any given component - volume density (V_V) - is expressed as a percentage of the total volume of the tissue. The volumes of CD4 and CD8 (Figure 4.2 and 4.3) immunopositive cells were calculated as percentage of the total tumour volume. A 100 point ocular grid was used at x 400 magnification and 30 fields were counted per case for each antibody. Only fields containing tumour tissues were counted. Any normal tissue on the slide was excluded from the analysis.

The volume density (V_V) of CD4 and CD8-positive lymphocytes was calculated for each tumour including tumour nest and stroma.

CD 68 and CD 34

Quantitative analysis of the tumour associated macrophage (Figure 4.4) / microvessel density (Figure 4.5) was by selecting three areas with high density of macrophages/neovascularisation (hot spots), where the highest numbers of macrophage/discrete vessels were stained at low power (4 x 10). Counting of

macrophage/discrete vessels was performed using a magnification of x200 (x20 objective and 1x10 ocular piece) using an ocular grid (10 x 10 square/ 0.25mm^2). Only fields within the tumour (including cancer cell nests and surrounding tissue stroma) were counted. Any normal tissue on the slide was excluded from the analysis.

COX-2

Semi-quantitative analysis of the COX-2 expression was scored using a weighted histoscore method. Histoscores were calculated from the sum of (1×% cells staining weakly positive)+(2×% cell staining moderately positive)+(3×% cells staining strongly positive) with a maximum of 300. Examples of positive staining patterns for cyclo-oxygenase-2 are shown Figure 4.6a, 4.6b and 4.6c.

All cases were counted by the author. For the purpose of assessing inter-observer reproducibility, a second observer (RC, AA) independently scored the slides. The agreement between observers was excellent (intra-class correlation coefficient (ICCC) (> 0.900) with no systematic differences. The observers were blinded to the clinical outcome of the patient.

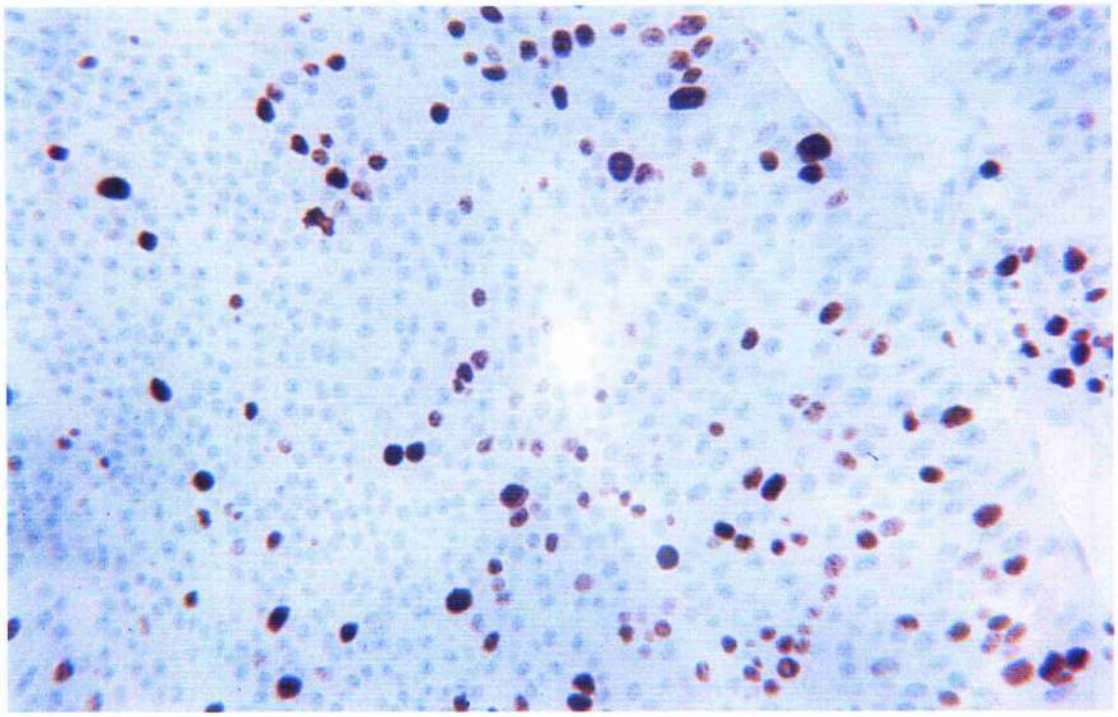

Figure 4.1 Immunohistochemical staining of Ki-67 antigen (brown) in transitional cell carcinoma of the urinary bladder

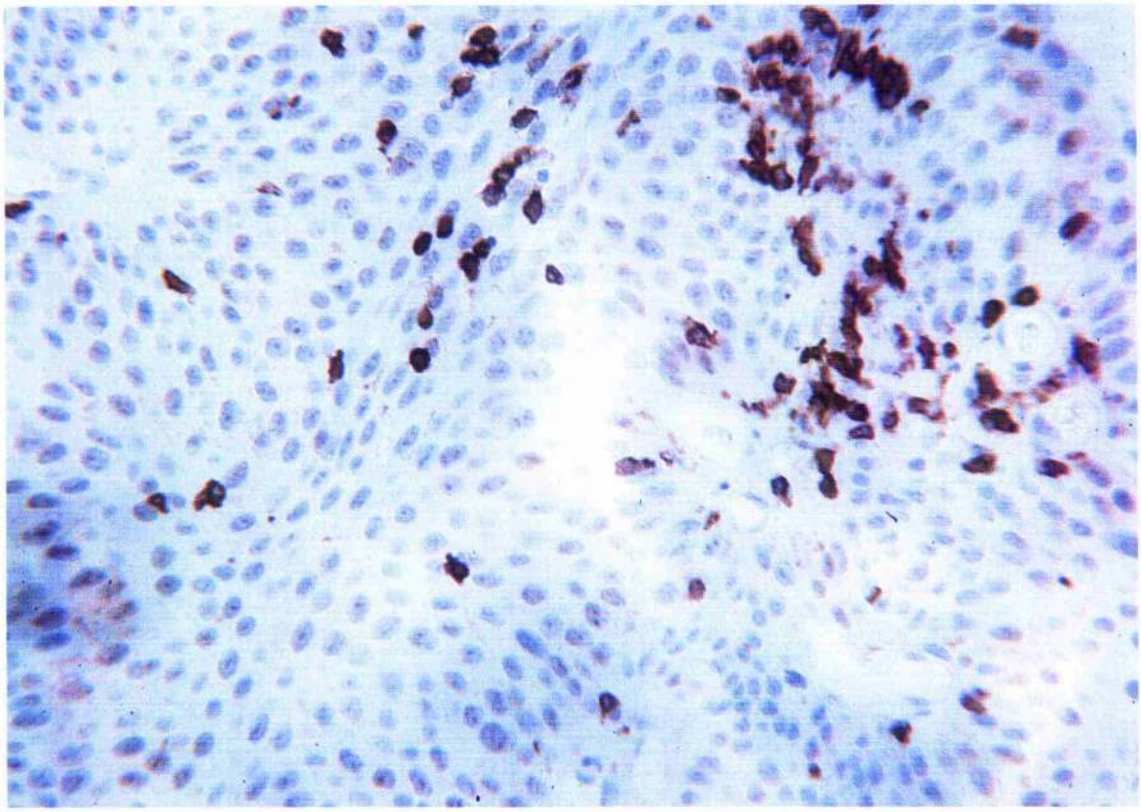

Figure 4.2 Immunohistochemical staining of CD4+ antigen (brown) in transitional cell carcinoma of the urinary bladder

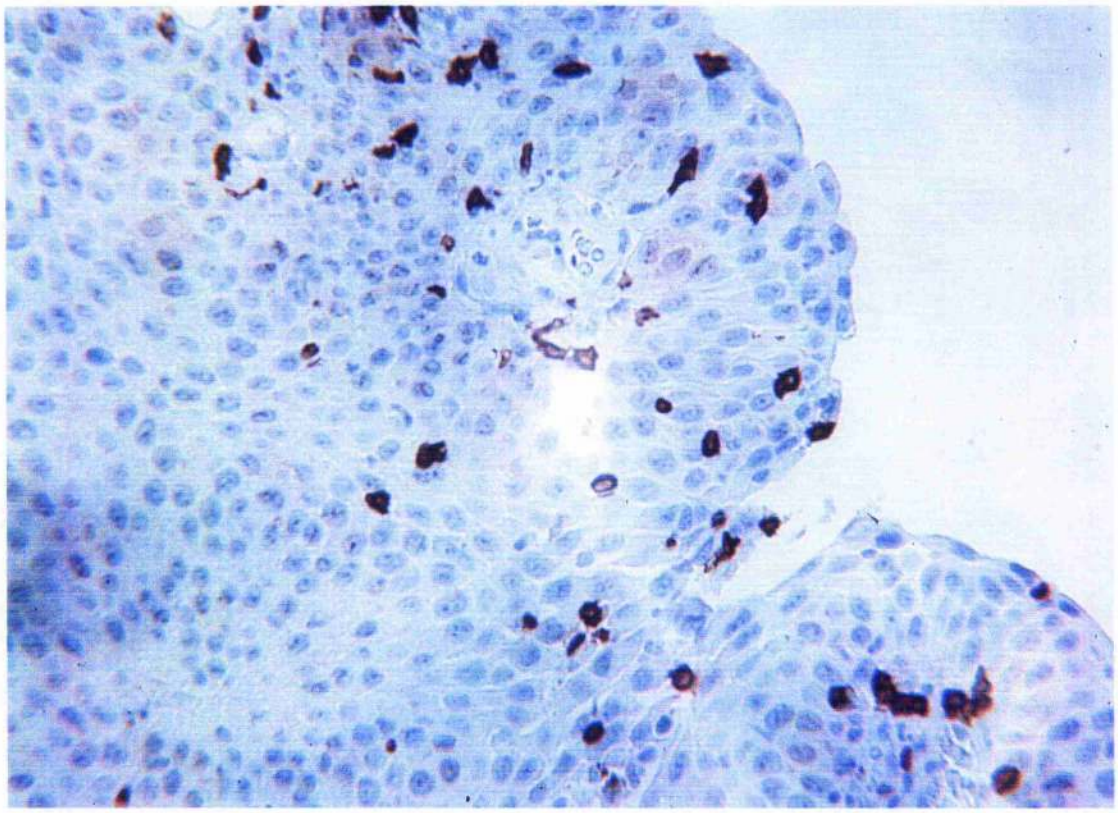

Figure 4.3 Immunohistochemical staining of CD8+ antigen (brown) in transitional cell carcinoma of the urinary bladder

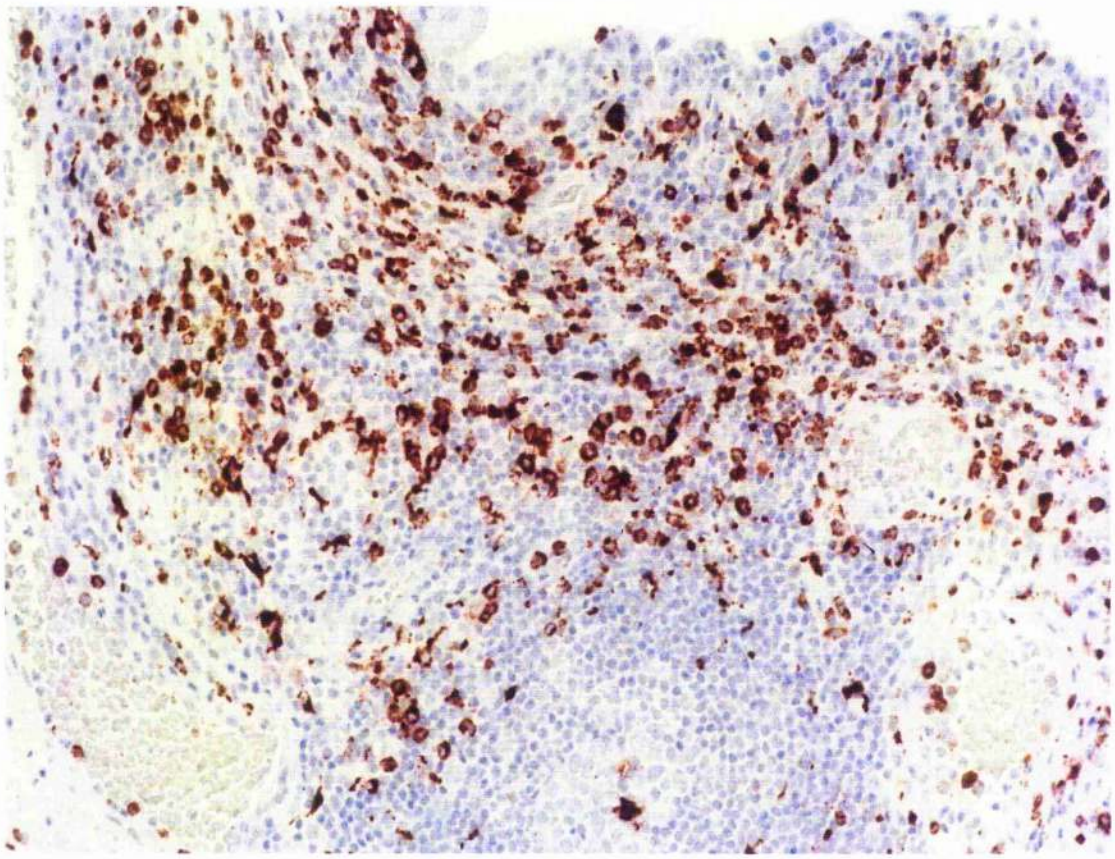

Figure 4.4 Immunohistochemical staining of CD 68+ antigen in transitional cell carcinoma of the urinary bladder

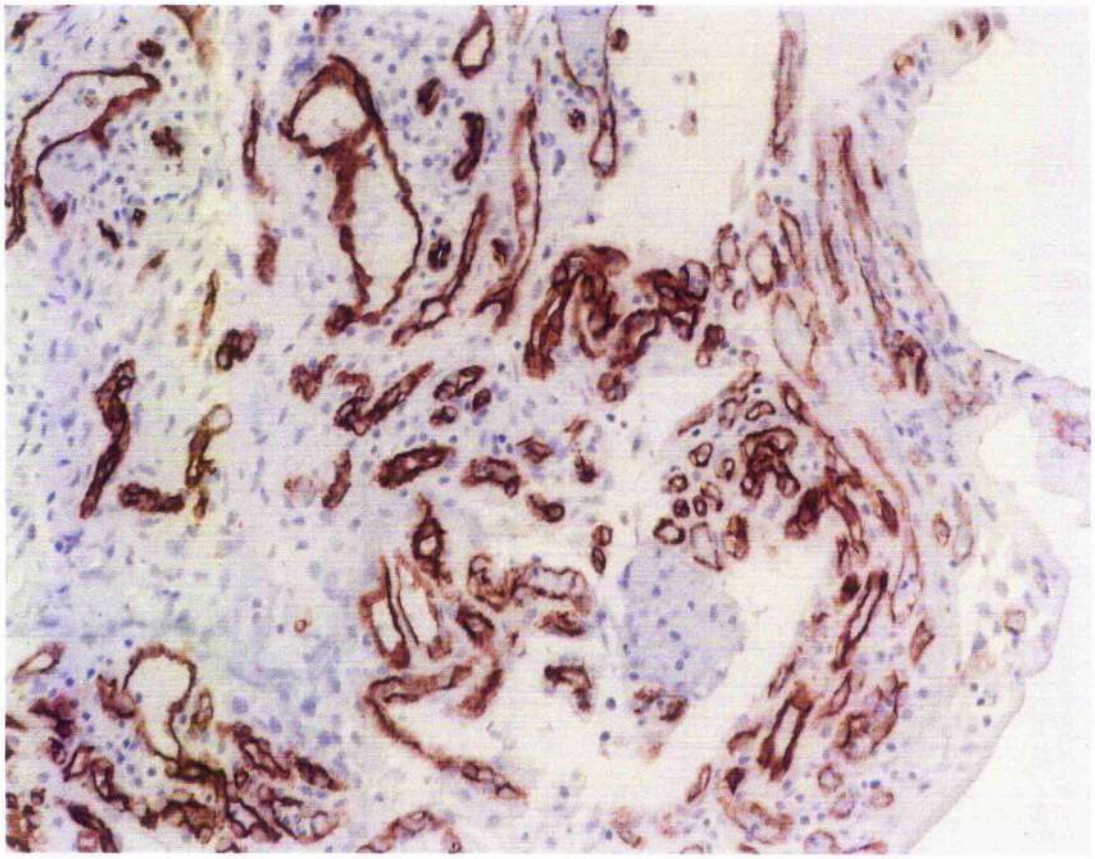

Figure 4.5 Immunohistochemical staining of CD 34+ antigen in transitional cell carcinoma of the urinary bladder

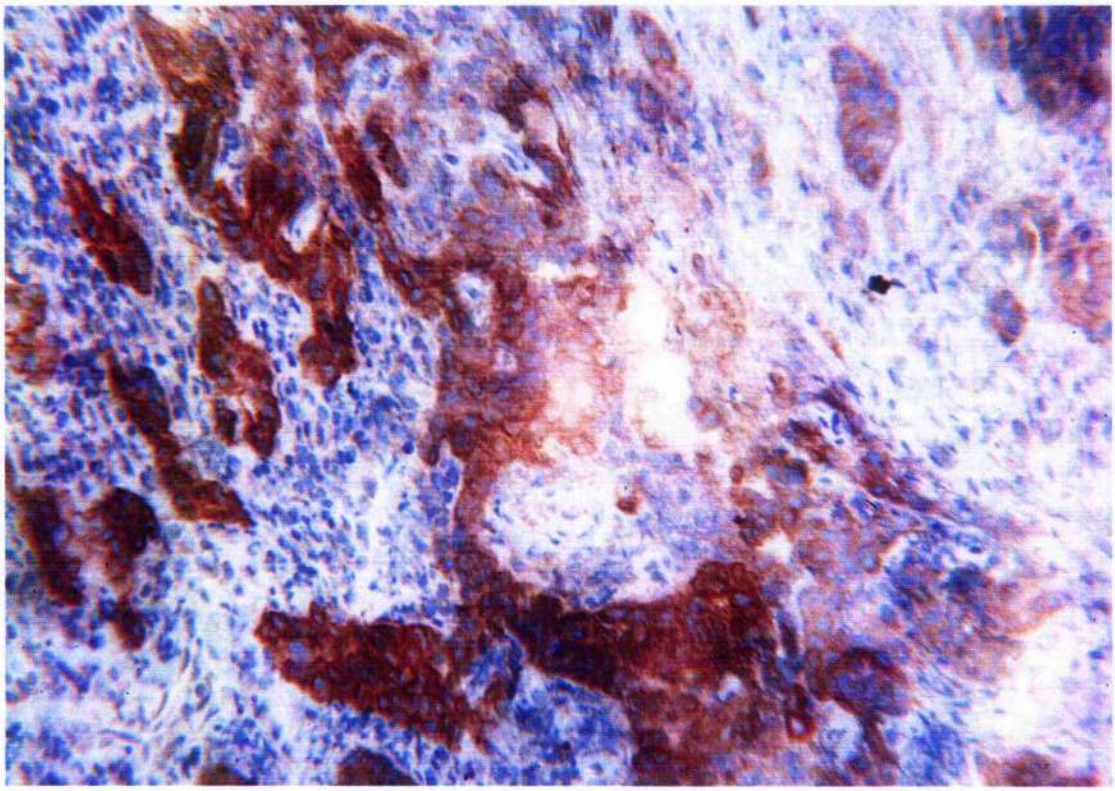

Figure 4.6 a Immunohistochemical staining of COX-2 antigen (brown) in transitional cell carcinoma of the urinary bladder – strong expression

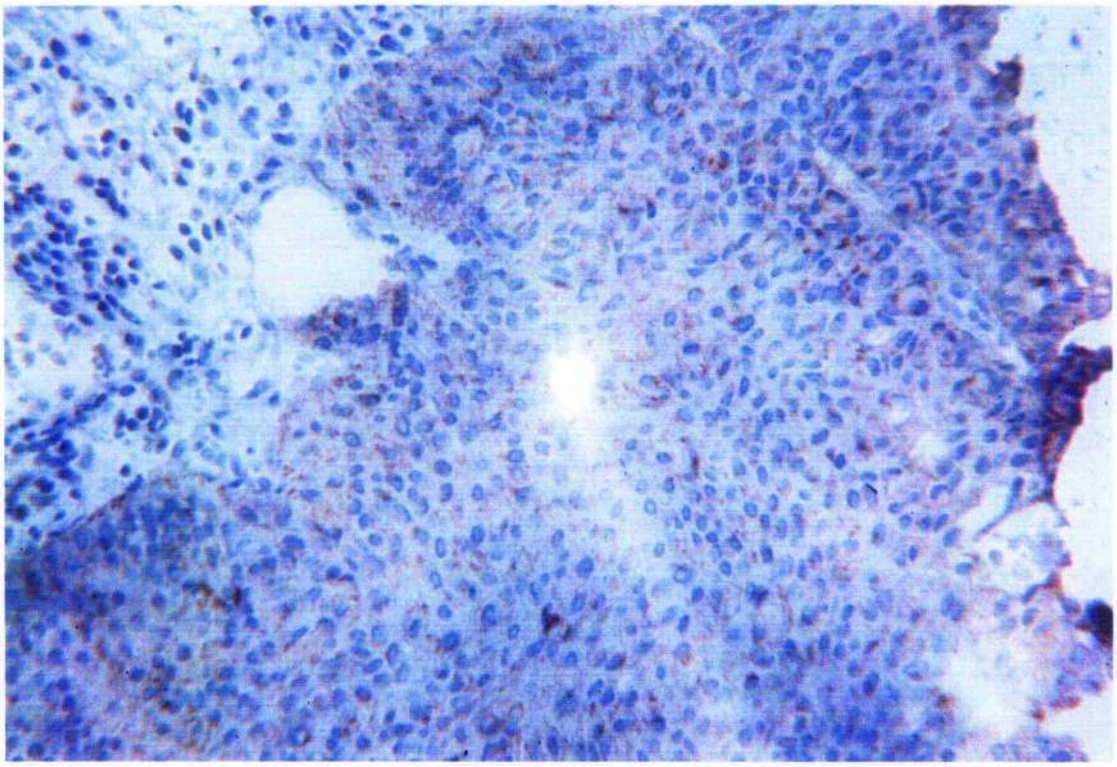

Figure 4.6 b Immunohistochemical staining of COX-2 antigen (brown) in transitional cell carcinoma of the urinary bladder – moderate expression

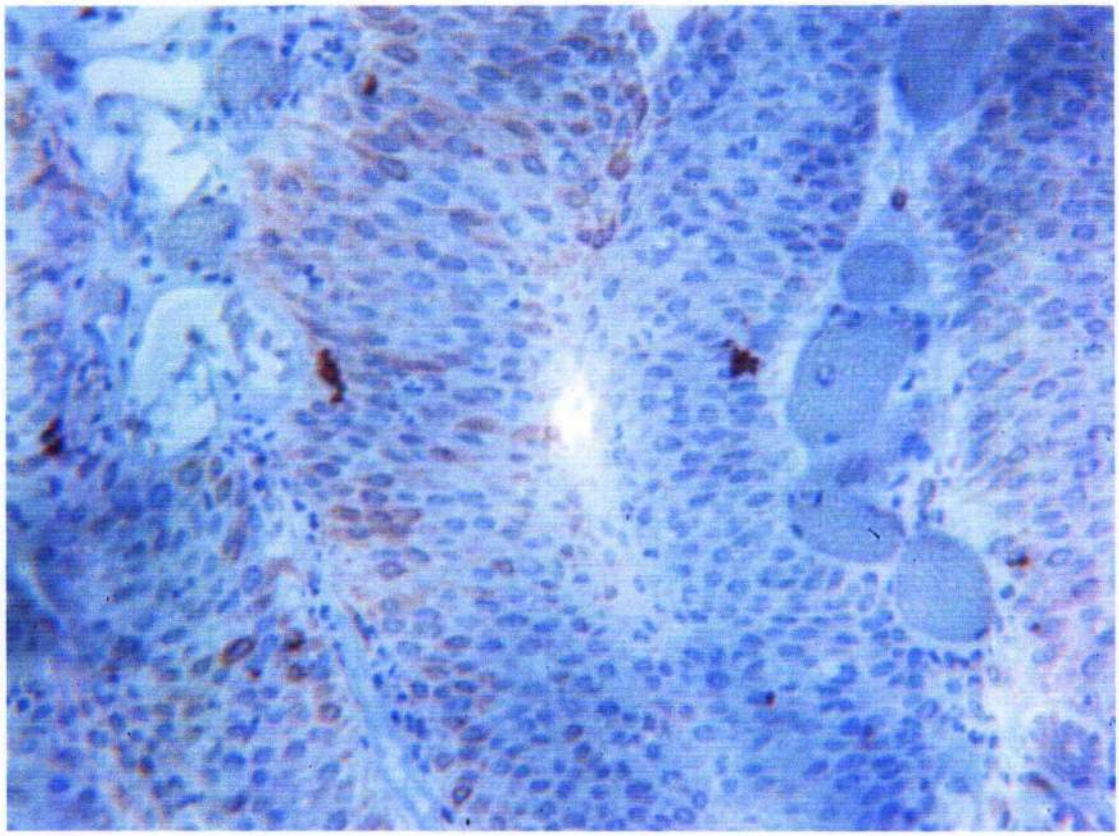

Figure 4.6 c Immunohistochemical staining of COX-2 antigen (brown) in transitional cell carcinoma of the urinary bladder – mild expression

4.3 Statistical Analysis

Data are presented as median and range. For the purpose of analysis the tumour Ki-67 labelling index, CD4+ and CD8+ T-lymphocyte counts, CD68+ and CD34+ and COX-2 expression were grouped by tertiles. The relationships between these and other variables were analysed using the Mantel-Haenszel (X^2) test for trend and Spearman rank correlation as appropriate.

Survival analysis was performed using the Cox proportional hazard model. Multivariate survival analysis was performed using stepwise backward procedure to derive a final model of the variables that had a significant independent relationship with survival. To remove a variable from the model, the corresponding P-value had to be a greater than 0.10. Analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA).

4.4 Results

The characteristics of patients with bladder cancer (n= 103) according to stage are shown in the Table 4.1. The majority of patients were male, over the age of 65 years, had superficial disease and had elevated C-reactive protein concentration pre-operatively. Patients with invasive bladder cancer were older ($p<0.01$), had higher grade tumours ($p<0.001$), elevated C-reactive protein concentrations ($p<0.05$) and an increased Ki-67 labelling index ($p<0.001$) compared with superficial disease. Also, patients with invasive disease had greater tumour infiltration of CD4+ ($p<0.05$) and CD8+ T-lymphocytes ($p<0.01$) and macrophages ($p<0.001$). Compared with superficial disease, patients with invasive bladder cancer had increased tumour microvessel density ($p<0.001$) and increased tumour expression of COX-2 ($p<0.001$). Patients with invasive bladder cancer received more adjuvant therapy ($p<0.05$). In total, 22 patients had adjuvant therapy (7 cystectomy, 9 radiotherapy and 6 BCG). Cancer-specific survival was shorter in those patients with invasive compared with superficial bladder cancer ($p<0.001$).

The minimum follow-up was 47 months; the median follow-up of the survivors was 60 months. During the course of the study, 66 patients died; 42 patients of their cancer and 24 of intercurrent disease. On univariate analysis, stratified by stage, elevated C-reactive protein ($p<0.05$), increased Ki-67 labelling index ($p<0.05$), increased tumour macrophage infiltration ($p<0.01$), increased tumour microvessel density ($p<0.001$), increased COX-2 expression ($p<0.05$) and adjuvant therapy ($p<0.01$) were associated with poorer cancer-specific survival (Table 4.2).

On multivariate analysis of these significant factors, stratified by stage, only elevated C-reactive protein (HR 2.47, 95%CI 1.18-5.18, $p=0.017$), increased tumour macrophage infiltration (HR 2.77, 95%CI 1.52-5.04, $p<0.001$) and adjuvant therapy

(HR 0.25, 95%CI (0.11-0.56, p<0.001) were independently associated with poorer cancer-specific survival. When C-reactive protein was excluded from the multivariate analysis, stratified by stage, only increased tumour macrophage infiltration (HR 2.71, 95%CI 1.50-4.91, p<0.001), increased tumour micro-vessel density (HR 1.63, 95%CI (1.00-2.64, p=0.049) and adjuvant therapy (HR 0.33, 95%CI (0.15-0.69, p=0.004) were independently associated with poorer cancer-specific survival.

The inter-relationships between the clinicopathological characteristics are shown in Table 4.3 and Figures 4.7a, 4 .7b and 4.7c. In all patients, tumour grade was directly associated with Ki-67 labelling index (p<0.001), CD4+ (p<0.01) and CD8+ (p<0.001) T-lymphocytes, CD68+ (p<0.001), CD34+ (p<0.01) and COX-2 expression (p<0.001), but not with C-reactive protein (p=0.152). An elevated C-reactive protein concentration was directly associated with the Ki-67 labelling index (p<0.05), CD68+ (p<0.001) and CD34+ (p<0.01). The Ki-67 labelling index was directly associated with CD4+ (p<0.001) and CD8+ (p<0.01) T-lymphocytes and CD68+ (p<0.001), CD34+ (p<0.001) and COX-2 expression (p<0.001). The tumour CD4+ T-lymphocytic infiltrate was directly associated with CD8+ (p<0.001), CD68+ (p<0.05), and COX-2 expression (p<0.01). The tumour CD8+ T-lymphocytic infiltrate was directly associated with COX-2 expression (p<0.05). The tumour CD68+ macrophage infiltrate was directly associated with CD34+ microvessel density (p<0.001) and COX-2 expression (p<0.001). The tumour CD34+ microvessel density was directly associated with COX-2 expression (p<0.001).

4.5 Discussion

In the present study, tumour grade and Ki-67 labelling index were closely associated with tumour T-lymphocytic and macrophage infiltrate, microvessel density and COX-2 expression. However, on multivariate analysis, when stratified for tumour stage, Ki-67 labelling index, macrophage infiltration and microvessel density but not T-lymphocytic infiltration or COX-2 expression were independently associated with cancer-specific survival. Furthermore, of these significant factors, tumour macrophage infiltration was the strongest. Taken together, the results of the present study would suggest that tumour macrophage infiltration is an important host response associated with disease progression and poor survival in patients with transitional cell carcinoma of the urinary bladder.

It was of interest that tumour macrophage infiltration was also associated with an elevated C-reactive protein concentration which was also independently associated with poorer cancer-specific survival. This relationship may reflect increased production, by macrophages, of interleukin-6 which is closely associated with liver production of C-reactive protein, a sensitive marker of the systemic inflammatory response (Gabay and Kushner, 1999). Taken together these observations would suggest that macrophage production, rather than tumour production, of interleukin-6 drives the systemic inflammatory response seen in bladder cancer.

These results are consistent with the report that an increase in interleukin-6 and its soluble receptor are associated with advanced tumour stage and predict disease recurrence and cancer-specific survival (Andrews et al., 2002). It remains to be determined whether the measurement of circulating interleukin-6 concentration offers superior prognostic value to that of either tumour macrophage infiltration or circulating C-reactive protein in patients with bladder cancer.

It was of interest that the tumour Ki-67 labelling index was associated with increased tumour infiltration by CD4+ and CD8+ T-lymphocytes in both superficial and invasive disease. These results are consistent with the concept that there is an active immune response to tumour cell proliferation in patients with transitional cell carcinoma of the urinary bladder and that bladder cancer is one of the few solid cancers to be treated successfully with immunotherapy. Indeed, intravesical treatment of superficial carcinoma with bacillus of Calmette and Guerin (BCG) vaccine reduces both disease recurrence and progression (Lamm et al., 2000). Furthermore, studies have shown that patients with marked lymphocytic infiltration have a better response to BCG (Alexandroff et al., 1999).

In summary, the results of the present study shows that, in addition to grade and Ki-67 labelling index, both local (CD68+ macrophages) and systemic (C-reactive protein) markers of the inflammatory response were associated with poor cancer-specific survival.

Table 4.1 The relationship between tumour stage and clinicopathological characteristics in patients with bladder cancer.

	Superficial (n= 61)	Invasive (n= 42)	P-value
Age group (<65/ >65)	30/ 31	10/ 32	0.010
Sex (male/ female)	44/ 17	26/ 16	0.277
Tumour grade			
G1/ G2/ G3	26/ 32/ 3	0/ 6/ 36	<0.001
C-reactive protein			
(<10/ >10mg/l)	32/ 29	13/ 29	0.031
% Tumour volume			
Ki-67 (tertiles 1, 2, 3)	30/ 20/ 11	5/ 14/ 23	<0.001
T-lymphocytes			
CD4+ (tertiles 1, 2, 3)	25/ 21/ 15	10/ 13/ 19	0.022
CD8+ (tertiles 1, 2, 3)	26/ 21/ 14	9/ 13/ 20	0.005
CD68+ (tertiles 1, 2, 3)	34 / 21 / 6	0 / 13 / 29	<0.001
CD34+ (tertiles 1, 2, 3)	29 / 20 / 12	5 / 14 / 23	<0.001
COX-2 (tertiles 1, 2, 3)	30/ 26/ 5	4/ 9/ 29	<0.001
Adjuvant therapy (no/ yes)	53/ 8	28/ 14	0.014
Cancer-specific survival			
(months)	109 (94-125)*	41 (24-58)*	<0.001

*Mean (95%CI)

Table 4.2 The relationship between clinicopathological characteristics and cancer-specific survival, stratified by stage, in patients with bladder cancer; univariate survival analysis.

	Patients (n= 103)	Hazard ratio (95%CI)	P-value
Age group (<65/ >65)	40/ 63	1.52 (0.76-3.02)	0.234
Sex (male/ female)	70/ 33	0.72 (0.37-1.39)	0.322
Tumour grade			
G1/ G2/ G3	26/ 38/ 39	1.18 (0.57-2.48)	0.653
C-reactive protein			
(<10/ >10mg/l)	45/ 58	2.36 (1.17-4.74)	0.016
% Tumour volume			
Ki-67 (tertiles 1, 2, 3)	34 (2-80)*	1.73 (1.11-2.72)	0.017
T-lymphocytes			
CD4+ (tertiles 1, 2, 3)	2.70 (0-12.30)*	1.26 (0.84-1.88)	0.260
CD8+ (tertiles 1, 2, 3)	2.20 (0.16-10.20)*	1.27 (0.87-1.86)	0.221
CD68+ (tertiles 1, 2, 3)	55 (18-98)*	2.48 (1.40-4.42)	0.002
CD34+ (tertiles 1, 2, 3)	49 (20-98)*	2.13 (1.27-3.57)	0.004
COX-2 (tertiles 1, 2, 3)	200 (100-300)*	1.93 (1.10-3.39)	0.023
Adjuvant therapy			
(no/ yes)	81/ 22	0.36 (0.17-1.75)	0.006

* median (range).

Table 4.3 The inter-relationships between the clinicopathological characteristics in patients with bladder cancer - Correlation co-efficient (p value).

	C-reactive protein (<10/ >10 mg/l)	Ki-67 labelling index (tertiles)	CD4+ (tertiles)	CD8+ (tertiles)	CD68+ (tertiles)	CD34+ (tertiles)	COX-2 (tertiles)
Tumour grade (G1/ G2/ G3)	0.15 (0.152)	0.49 (0.001)	0.25 (0.009)	0.38 (<0.001)	0.54 (<0.001)	0.30 (0.004)	0.56 (<0.001)
C-reactive protein (<10/ >10 mg/l)		0.20 (0.039)	-0.06 (0.556)	0.01 (0.892)	0.39 (<0.001)	0.49 (0.001)	0.17 (0.089)
Ki-67 labelling Index (tertiles)			0.45 (<0.001)	0.30 (0.002)	0.45 (<0.001)	0.35 (<0.001)	0.56 (<0.001)
CD4+ (tertiles)				0.41 (<0.001)	0.20 (0.040)	0.00 (0.999)	0.29 (0.003)
CD8+ (tertiles)					0.16 (0.107)	0.17 (0.079)	0.23 (0.018)
CD68+ (tertiles)						0.52 (<0.001)	0.57 (<0.001)
CD34+ (tertiles)							0.48 (<0.001)

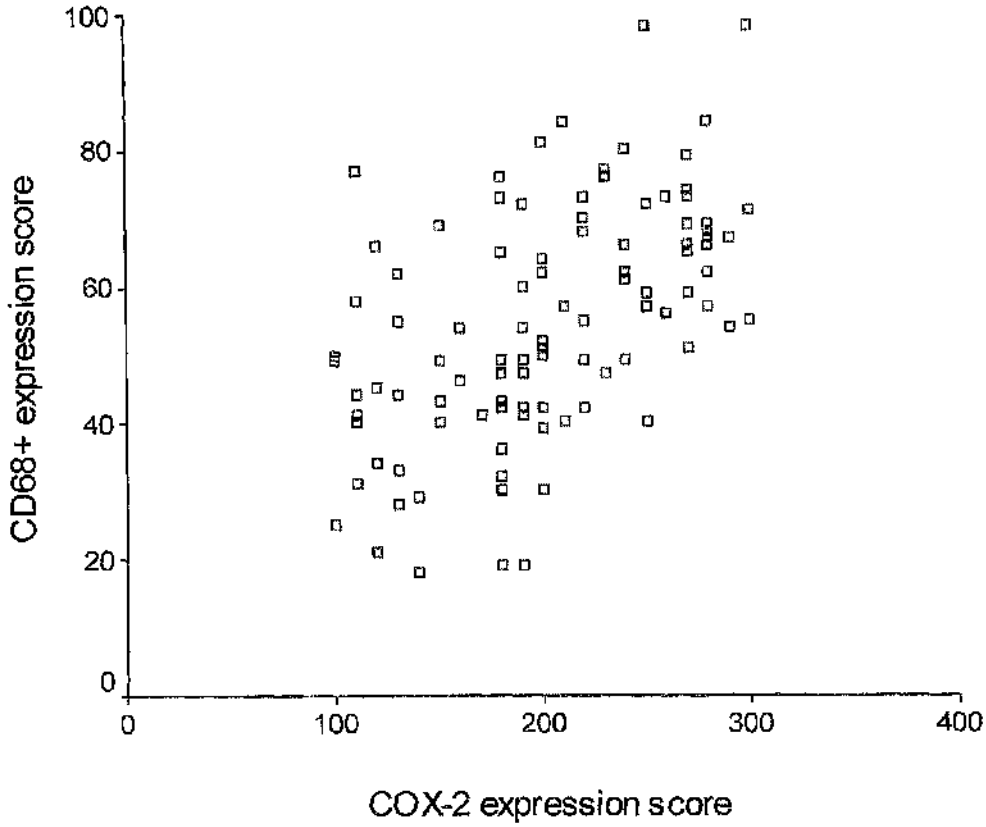

Figure 4.7a The relationship between CD68+ (tumour associated macrophage, TAM) and COX-2 expression ($p < 0.001$) in patients with transitional cell carcinoma of the urinary bladder.

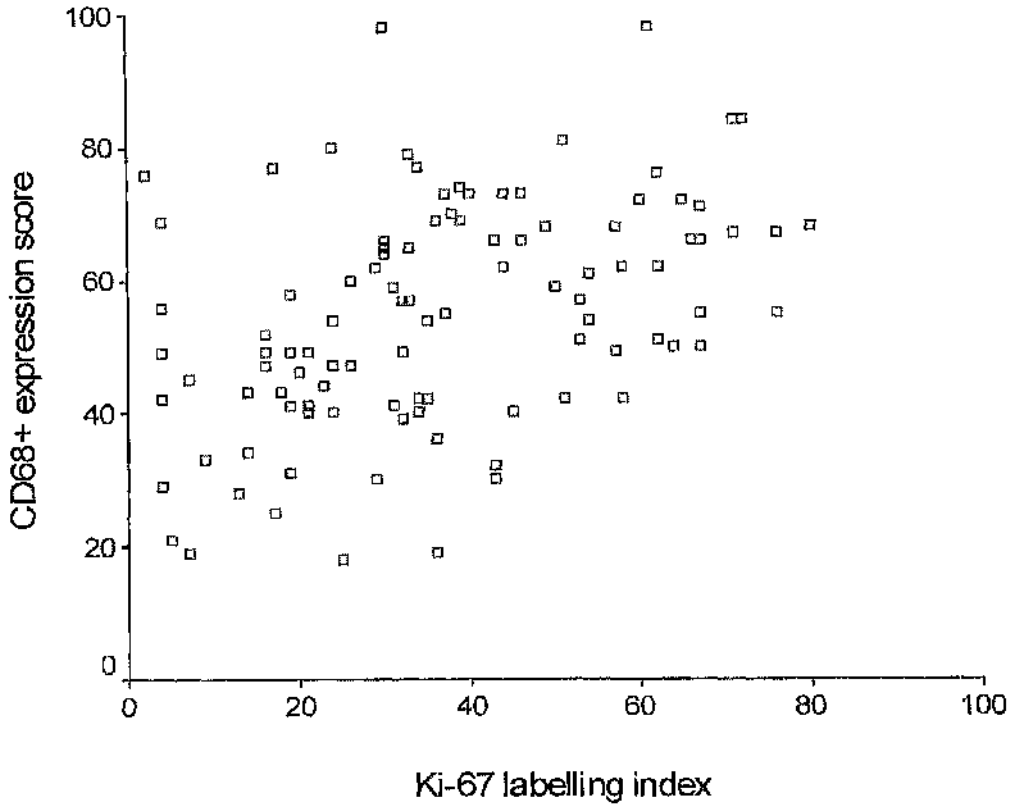

Figure 4.7b The relationship between CD68+ (tumour associated macrophage, TAM) and Ki-67 proliferative index ($p < 0.001$) in patients with transitional cell carcinoma of the urinary bladder.

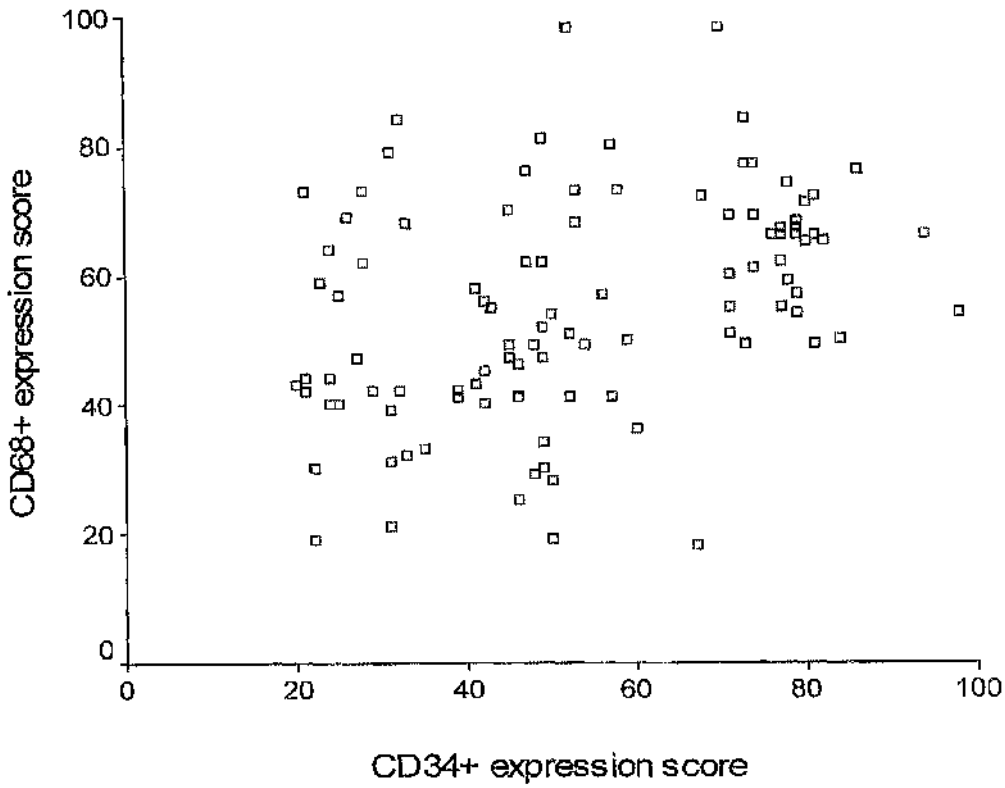

Figure 4.7c The relationship between CD68+ (tumour associated macrophage, TAM) and CD34+ expression (microvessel density), ($p < 0.001$) in patients with transitional cell carcinoma of the urinary bladder.

CHAPTER 5: THE RELATIONSHIP BETWEEN THE SYSTEMIC INFLAMMATORY RESPONSE AND CYTOKINES/ CHEMOKINES PROFILE IN PATIENTS WITH TRANSITIONAL CELL CARCINOMA OF THE URINARY BLADDER.

5.1 Introduction:

It is increasingly recognised that, in addition to tumour stage and proliferative activity, disease progression is dependent on a complex interaction of the tumour and host inflammatory response (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). Indeed, in chapter 3 we have shown that, in addition to stage and grade, the systemic inflammatory response, as evidenced by elevated circulating concentrations of C-reactive protein, was associated with poor cancer-specific survival.

It is now recognised that elevated circulating concentrations of C-reactive protein reflect the net effect of hormones and cytokines/chemokines (Gabay and Kushner, 1999). To date, few studies have examined the cytokines/chemokines profile associated with an elevated C-reactive protein concentration in patients with cancer. Such profiles may be useful in identifying which immune cells are activated and play a role in the elaboration of C-reactive protein. The aim of the present study was to examine the relationship between circulating concentrations of C-reactive protein and cytokines produced by T-lymphocyte subset populations in patients with transitional cell carcinoma of the bladder.

5.2 Methods:

A prospective study of newly diagnosed patients with transitional cell carcinoma of the bladder at hospitals in the North Glasgow Trust was studied. Prior to elective admission for transurethral resection of the bladder tumour, blood samples were taken for routine laboratory measurement of C-reactive protein measurement and plasma storage for cytokines/ chemokines profile measurement.

Inclusion criteria were newly-diagnosed patients with transitional cell carcinoma of the bladder and signed informed consent. Exclusion criteria were patients with a known systemic inflammatory condition e.g. inflammatory bowel disease, rheumatoid arthritis or known infection at the time of entry into the study. Following discussion of the study with the patient and completion of the consent form, two 4ml blood samples were taken prior to the transurethral resection of the bladder tumour. The samples were centrifuged and the plasma is frozen and stored at -20 degrees.

Tumours were divided into two groups, superficial (pTa, pT1 and CIS) and muscle invasive (pT2-pT4). However, patients with pT1 G3 were grouped with the muscle-invasive tumours since such tumour have a significant higher progression rate (Manoharan and Soloway, 2005). At this time no patient showed clinical evidence of infection, or other inflammatory conditions. Tumour stage was assessed using the 1997 AJCC/UICC TNM classification (Sobin and Wittcking, 2002) and tumour grade performed according to the 1999 WHO grading system (Busch and Algaba, 2002). The Research Ethics Committee of North Glasgow NHS Trust approved the study.

The limit of detection of the assay for C-reactive protein was 5 mg/l. The coefficient of variation, over the range of measurement, was less than 5% as established by

routine quality control procedures. C-reactive protein measurement of greater than 10mg/l was considered to indicate the presence of systemic inflammatory response (O'Gorman et al., 2000).

Frozen plasma samples stored at –20°C were thawed once and 50 µl was used to measure cytokine levels utilising the novel Luminex technology, a development on multiplex assays first described in 1977 (Horan and Wheeless, 1977). Luminex technology utilises patented pre-dyed microspheres allowing up to 100 analytes to be measured simultaneously in the one biological sample. Briefly, an individual bead set is coated with a capture antibody against the analyte (cytokine) of choice. Combinations of these bead sets are then incubated with sample and thereafter a cocktail of detection antibodies labelled with a reporter flourochrome is added similar to a conventional sandwich ELISA. Detection of beads and quantification of analytes was performed using a Multiplex system and Bio-Plex software (Bio-Rad, California, USA).

In the present study the analysis was carried out as per manufacturer's instruction using preconfigured multiplex cytokine kits (Biosource, California, USA).

From examination of the cytokine data and the report of de Jager and co-workers (2003) it was clear that the sensitivity was limited to 5pg/ml in this assay system and therefore cytokines/chemokines concentrations measured below this limit were set to 5pg/ml.

5.3 Statistical Analysis

Data are presented as median and range. Inflammatory cytokines/chemokines profile concentration below the threshold of sensitivity of the respective assays were expressed as equal to this threshold. Where appropriate, data were tested for statistical significance using the Mann-Whitney U test. Analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA).

5.4 Results:

The characteristics of patients with bladder cancer (n= 47), grouped according to the presence and absence of a systemic inflammatory response, are shown in Table 1. The majority of patients were male, over the age of 65 years, had superficial disease and a C-reactive protein concentration in the normal range. In total, 16 patients had adjuvant therapy (7 cystectomy, 2 radiotherapy and 7 BCG).

Of the Th0 type cytokines/chemokines measured, IL-6, IL-7, IL-8, IL-15 and GM-CSF had median concentrations below the limit of detection (5pg/ml) irrespective of their C-reactive protein concentrations, tumour stage and grade. IL-1b, and IL-12 had similar detectable concentrations irrespective of their C-reactive protein concentrations, tumour stage and grade.

Of the Th1 type cytokines/chemokines measured, IL-2, interferon-gamma and TNF-alpha had median concentrations below the limit of detection (5pg/ml) irrespective of their C-reactive protein concentrations, tumour stage and grade.

Of the Th2 type cytokines/chemokines measured, IL-4, IL-5, IL-10 and IL-13 had median concentrations below the limit of detection (5pg/ml) irrespective of their C-reactive protein concentrations, tumour stage and grade.

Of the macrophage/monocyte-type cytokines/chemokines (MIP-1 alpha, MIP-1 beta and MCP-1) and eosinophils-type cytokines/chemokines (EOTAXIN) had detectable median plasma concentrations but also were not associated with elevated C-reactive protein concentrations, tumour stage and grade apart from MIP-1 beta which was associated with tumour stage ($p=0.043$). These findings were inconclusive because of the low concentrations measured in the plasma.

5.5 Discussion

It is well recognised that cytokines are secreted by and are regulators of immune system. Furthermore, different cytokine functions are complex and overlapping and therefore this means that the analysis of multiple related cytokines is more informative than the measurement of a single cytokine.

In the present study we wished to examine the circulating concentrations of those cytokines recognised to have a role in immune function. It was anticipated that the cytokines/ chemokines profile would give further information as to the nature of the immune response in patients with bladder cancer. We have previously shown that circulating cytokine concentrations differ between those patients with and without elevated C-reactive protein concentrations using an ELISA system for single cytokines (McKeown et al., 2004). In the present study, although the assay system used was capable of measuring 21 cytokines at the same time, the sensitivity was limited and the majority of samples had undetectable concentrations. The result of this was that there appeared to be no significant difference in cytokine concentrations between those patients with and without elevated C-reactive protein concentrations.

To date multiplex analysis of cytokines has been almost exclusively limited to cell culture supernatants. In terms of the number of peptides and proteins such supernatants are much less complex than that of a plasma or serum sample. Indeed, the problems of analysing plasma or serum samples have been suggested by De Jager and colleagues (2003) who note that when sera are used as the analytical matrix the matrix should be carefully monitored for blocking substances such as heterophilic antibodies. Taken together the

above observations would suggest that there is a need to pre-treat plasma or serum samples prior to multiplex analysis of cytokines.

In summary there remain a number of technical challenges to be overcome before multiple cytokine analysis can be applied to identify the nature of the immune response in patients with a systemic inflammatory response and transitional cell carcinoma of the urinary bladder.

Table 5.1 Clinicopathological characteristics and cytokines/ chemokines profiles according to the presence or absence of a systemic inflammatory response in patients with transitional cell carcinoma of the urinary bladder.

	No systemic inflammatory response CRP ≤10mg/l (n=31)	Systemic inflammatory response CRP >10mg/l (n=16)	p-value
Age (≤65/ >65yrs)	9/ 22	6/ 10	0.559
Sex (male/ female)	23/ 8	11/ 5	0.696
Stage (superficial/ invasive)	22/ 9	9/ 7	0.318
Grade (G1/ G2/ G3)	4/ 12/ 15	3/ 8/ 5	0.295
Th0 cytokines			
IL-1b	35 (<5-1465)	35 (<5-1422)	0.917
IL-6	<5 (<5-2586)	6 (<5-173)	0.613
IL-7	<5 (<5-590)	<5 (<5-172)	0.915
IL-8	<5 (<5-198)	<5 (<5-46)	0.574
IL-12	49 (27-742)	43 (18-760)	0.606
IL-15	<5 (<5-613)	<5 (<5-828)	0.800
GM-CSF	<5 (<5-4132)	<5 (<5-106)	0.516
RANTES	17572 (<5-132605)	19374 (1283-539602)	0.928
Th1 cytokines			
IL-2	<5 (<5-1960)	<5 (<5-1469)	0.279
INF gamma	<5 (<5-<5)	<5 (<5-17)	0.164
TNF alpha	<5 (<5-236)	<5 (<5-863)	0.974
IP-10	34 (7-204)	44.5 (23-80)	0.381
MIG	<5 (<5-361)	<5 (<5-576)	0.55

continue Table 5.1

	No systemic inflammatory response	Systemic inflammatory response	P value
Th2 cytokines			
IL-4	<5 (<5-201)	<5 (<5-166)	0.487
IL-5	<5 (<5-53)	<5 (<5-<5)	0.304
IL-10	<5 (<5-1216)	<5 (<5-19)	0.907
IL-13	<5 (<5-75)	<5 (<5-33)	0.937
Eosinophil cytokines			
EOTAXIN	58 (<5-172)	69.5 (14-219)	0.728
Monocyte/macrophage cytokines			
MIP-1 alpha	33 (<5-132)	36.5 (8-705)	0.253
MIP-1 beta	39 (<5-488)	66 (<5-2044	0.291
MCP-1	120 (66-1500)	137 (63-691)	0.261

Median (range)

CHAPTER 6: CONCLUSION

6.1 Introduction

In chapter two, the aims of this thesis were defined as follows:

To investigate the following in patients with transitional cell carcinoma of the urinary bladder:

1. The prognostic value of the systemic inflammatory response.
2. The relationship between the systemic inflammatory response, tumour proliferative activity, T-lymphocytic infiltration, COX-2 expression, tumour associated macrophage and microvessel density and survival.
3. The relationship between the T-lymphocyte cytokine profile and the systemic inflammatory response.

6.2 Summary of the findings

In chapter three, we have shown that in common with those suffering a wide variety of solid tumours, patients with transitional cell carcinoma of the urinary bladder mount a systemic inflammatory response as evidenced by raised circulating concentration of C-reactive protein, independent of tumour-based factors e.g. stage and grade.

In chapter four, several findings were observed: there was a strong correlation between tumour grade and tumour proliferative index and both were closely associated with tumour T-lymphocytic and macrophage infiltrate, microvessel density and COX-2 expression. In addition, tumour stage, Ki-67 labelling index, macrophage infiltration and microvessel density but not T-lymphocytic infiltration or COX-2 expression were independently associated with cancer-specific survival. This would suggest that tumour

macrophage infiltration is an important host response associated with disease progression and poor survival in patients with transitional cell carcinoma of the urinary bladder. Moreover, this would suggest that macrophage production, rather than tumour production, of interleukin-6 drives the systemic inflammatory response seen in bladder cancer.

In chapter five, we anticipated that the cytokine profile would inform as to whether a Th1 or Th2 response was dominant in patients with bladder cancer. However, although the assay system used was capable of measuring 14 cytokines at the same time the sensitivity was limited and the majority of samples had undetectable concentrations. The result of this was that there appeared to be no significant difference in cytokine concentrations between those patients with and without elevated C-reactive protein concentrations, tumour stage and grade.

In the present studies there are a number of potential biases. These include the following: that the cohort was retrospective and that both superficial and invasive disease patients were studied. However, it is clear that the study cohort was representative, in terms of clinical pathological characteristics, of a larger cohort which presented to the Glasgow Royal Infirmary between 1992 and 1999. With regard to superficial and invasive disease, it would have been better to consider these patient groups separately. Moreover, although cancer-specific survival is a strong end-point it might have been better to have considered relapse-free survival in those patients with superficial disease. However, to account for such a bias, the patients were stratified according to stage in our analysis.

It is clear from recent studies that bladder cancer is a heterogeneous disease. For example, superficial disease differs from invasive disease in that, in the latter, there is high incidence of disease recurrence and progression. There are a number of molecular differences between these disease states. For example, oncogenes thought to be important in the progression of bladder cancer, from superficial to invasive disease, include HER2/neu (Miyamoto et al., 2000). Moreover, several investigators have shown a positive association between over-expression of epidermal growth factor receptor (EGFR) and high-grade, high-stage bladder cancer and poor survival (Lipponen et al., 1994). In addition, apoptotic index and Ki-67 labelling index appears to correlate with tumour grade and stage (Gonzalez-Campora et al., 2006). Matrix metalloproteinases are intimately involved in tumour-associated degradation of the extra cellular matrix and one of these factors, MMP-9; expression was directly related to increasing tumour stage (Guan et al., 2003). However, the functional biological relationship between these prognostic factors remains unclear. Such differences could be secondary to genetic instability and accumulation of collaborative genetic lesions mainly involving p53, retinoblastoma (Rb), and growth factors (Al-Sukhun and Hussain, 2003).

6.3 Significance of findings

It would appear that the systemic inflammatory response is an independent prognostic factor in patients with bladder cancer. These results are consistent with the superior prognostic value of C-reactive protein compared with tumour T-lymphocytic infiltration in patients with primary operable colorectal cancer. One possible explanation is that C-reactive protein can be measured with greater accuracy and

precision than tumour-based factors. Alternatively, it may be that C-reactive protein plays a more pivotal role in the tumour-host relationship. Indeed, there is increasing evidence that C-reactive protein has prognostic value, independent of the malignant potential, in a variety of tumours. The role of the systemic inflammatory response in determining disease-specific survival in patients with bladder cancer is worthy of further study to establish its value as a prognostic index. Such an index would help stratify patients into groups at the time of diagnosis and staging into three groups. The first is the group with the least chance of recurrence and progression, which will require less frequent and shorter period of follow-up. The middle group would be followed as per the standard follow-up schedule (3 monthly cystoscopy for the first year followed by 6 monthly for 2 years then annually thereafter for life). The third is the group with the highest chance of recurrence and progression, which will require more frequent follow-up and more aggressive treatment.

Such a role needs to be verified in a larger prospective study with a mature follow-up. The process of assessing these inflammatory markers routinely will require some investment in such investigative tools e.g. the use of quantitative image analysis.

This thesis supports the measurement of C-reactive protein in patients with bladder cancer as a prognostic marker. Moreover, the findings in this thesis support future study of the use of anti-inflammatory agents e.g. Ibuprofen, as a means of modifying the inflammatory response and so perhaps improving survival in patients with transitional cell carcinoma of the urinary bladder.

The observation that there is a correlation between tumour grade and tumour proliferative index is of importance. Assessing the tumour proliferative index in addition to tumour grade would be useful. This could be a useful tool to identify the

correct tumour grade especially in grade 2 tumours in which there is a high inter-observer difference in grading.

In superficial disease, recurrence and progression would be clinically relevant since 70% of patients with superficial disease will have recurrence and of these 50% within 2 years ((Zieger et al., 1998). Of these recurrences 20% will progress to an invasive disease. Approximately 10% of patients would die from their superficial cancer compared with approximately 50% of patients with invasive disease. Therefore, in superficial disease, future studies of the prognostic value of markers of the local/ systemic inflammatory response should also include recurrence and progression as end-points.

The bladder as a confined compartment, in which high local concentrations of the immunotherapeutic agent and effective recruitment of immune cells can be achieved, serves as an ideal target organ for this type of immunotherapeutic approach. Thalidomide is associated with high levels of TNF, raised CD4+ and suppresses angiogenesis, and studies suggest that the BCG effect on bladder cancer may be through TNF (Alexandroff et al., 1999). Therefore, giving intravesical thalidomide may help to control the disease and may not have the systemic side effects of thalidomide.

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APPENDIX 2: CLINICOPATHOLOGICAL DATA (CHAPTER 4)

There follows the raw data collected for the retrospective study for patients with transitional cell carcinoma of the urinary bladder which is discussed in chapter 4.

Abbreviations Used in Tables

Treatment Code:

0 = Transurethral resection of bladder tumour + single intravesical mitomycin

1 = Cystectomy

2 = Radical radiotherapy

3 = Intravesical BCG

Follow-up Code:

0 = Alive

1 = Bladder cancer specific death

2 = Death due to intercurrent disease

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Age	Age Code ≤ 65/ >65yr	Sex	Preoperative CRP mg/l	CRP Code (preop) ≤ 10/ > 10mg/l	Postoperative CRP mg/l	CRP Code (postop) ≤ 10/ > 10mg/l
1	66.17	1.00	m	102.00	1.00		
2	54.81	.00	m	49.00	1.00	47.00	1.00
3	67.13	1.00	m	17.00	1.00		
4	79.75	1.00	m	36.00	1.00		
5	72.60	1.00	m	46.00	1.00	145.00	1.00
6	54.27	.00	m	11.00	1.00	8.00	.00
7	65.43	.00	f	31.00	1.00	59.00	1.00
8	75.84	1.00	f	25.00	1.00	106.00	1.00
9	80.35	1.00	f	5.00	.00		
10	65.86	.00	m	15.00	1.00	10.00	.00
11	72.88	1.00	f	37.00	1.00		
12	79.70	1.00	m	5.00	.00	5.00	.00
13	71.43	1.00	f	152.00	1.00		
14	62.20	.00	m	5.00	.00	5.00	.00
15	63.30	.00	m	37.00	1.00		
16	72.31	1.00	m	98.00	1.00		
17	75.84	1.00	m	5.00	.00	15.00	1.00

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Age	Age Code ≤ 65/ >65yr	Sex	Preoperative CRP mg/l	CRP Code (preop) ≤ 10/ > 10mg/l	Postoperative CRP mg/l	CRP Code (postop) ≤ 10/ > 10mg/l
18	57.31	.00	m	5.00	.00	9.00	.00
19	61.30	.00	f	30.00	1.00	8.00	.00
20	72.62	1.00	f	165.00	1.00		
21	64.76	.00	m	29.00	1.00		
22	73.77	1.00	m	12.00	1.00		
23	59.98	.00	m	92.00	1.00	25.00	1.00
24	80.96	1.00	m	159.00	1.00	5.00	.00
25	63.32	.00	f	34.00	1.00		
26	86.15	1.00	f	48.00	1.00		
27	60.50	.00	m	46.00	1.00	88.00	1.00
28	68.01	1.00	m	25.00	1.00	21.00	1.00
29	69.25	1.00	m	13.00	1.00		
30	75.13	1.00	m	11.00	1.00		
31	65.30	.00	m	229.00	1.00		
32	67.87	1.00	m	16.00	1.00	44.00	1.00
33	72.30	1.00	m	5.00	.00		
34	77.85	1.00	m	9.00	.00	5.00	.00

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Age	Age Code ≤ 65/ >65yr	Sex	Preoperative CRP mg/l	CRP Code (preop) ≤ 10/ > 10mg/l	Postoperative CRP mg/l	CRP Code (postop) ≤ 10/ > 10mg/l
35	68.67	1.00	m	6.00	.00		
36	79.63	1.00	m	5.00	.00	54.00	1.00
37	68.95	1.00	m	5.00	.00		
38	65.06	.00	m	121.00	1.00	10.00	.00
39	64.82	.00	m	5.00	.00	5.00	.00
40	68.37	1.00	m	157.00	1.00		
41	58.04	.00	m	16.00	1.00	17.00	1.00
42	66.19	1.00	m	5.00	.00	5.00	.00
43	82.34	1.00	m	5.00	.00	38.00	1.00
44	65.41	.00	f	5.00	.00	21.00	1.00
45	61.29	.00	f	5.00	.00	18.00	1.00
46	77.90	1.00	m	98.00	1.00		
47	57.47	.00	m	5.00	.00	5.00	.00
48	79.45	1.00	m	42.00	1.00	63.00	1.00
49	54.29	.00	m	38.00	1.00		
50	64.79	.00	f	5.00	.00	27.00	1.00
51	64.95	.00	f	14.00	1.00		

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Age	Age Code ≤ 65/ >65yr	Sex	Preoperative CRP mg/l	CRP Code (preop) < 10/ > 10mg/l	Postoperative CRP mg/l	CRP Code (postop) < 10/ > 10mg/l
52	57.89	.00	f	8.00	.00	5.00	.00
53	90.47	1.00	f	135.00	1.00		
54	63.42	.00	m	15.00	1.00	95.00	1.00
55	56.09	.00	m	153.00	1.00		
56	50.54	.00	m	5.00	.00	10.00	.00
57	73.30	1.00	f	22.00	1.00	166.00	1.00
58	71.99	1.00	m	5.00	.00		
59	43.56	.00	f	40.00	1.00	198.00	1.00
60	73.90	1.00	m	68.00	1.00		
61	86.83	1.00	f	5.00	.00	5.00	.00
62	85.00	1.00	m	5.00	.00	5.00	.00
63	57.58	.00	f	5.00	.00	5.00	.00
64	74.83	1.00	f	5.00	.00	7.00	.00
65	80.60	1.00	m	194.00	1.00		
66	84.24	1.00	m	5.00	.00		
67	93.02	1.00	m	5.00	.00	5.00	.00
68	68.12	1.00	m	30.00	1.00		

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Age	Age Code ≤ 65/ >65yr	Sex	Preoperative CRP mg/l	CRP Code (preop) < 10/ > 10mg/l	Postoperative CRP mg/l	CRP Code (postop) < 10/ > 10mg/l
69	79.69	1.00	m	34.00	1.00	57.00	1.00
70	64.63	.00	m	24.00	1.00		
71	78.06	1.00	f	55.00	1.00	108.00	1.00
72	73.90	1.00	m	5.00	.00		
73	91.28	1.00	m	37.00	1.00	5.00	.00
74	84.48	1.00	f	10.00	.00	5.00	.00
75	72.25	1.00	m	88.00	1.00	277.00	1.00
76	52.25	.00	f	8.00	.00	8.00	.00
77	60.77	.00	f	5.00	.00	5.00	.00
78	72.75	1.00	m	5.00	.00		
79	59.84	.00	m	11.00	1.00	11.00	1.00
80	70.73	1.00	f	5.00	.00	5.00	.00
81	82.68	1.00	m	5.00	.00	19.00	1.00
82	74.47	1.00	m	62.00	1.00		
83	64.81	.00	m	5.00	.00	5.00	.00
84	79.56	1.00	f	5.00	.00	27.00	1.00
85	68.01	1.00	m	26.00	1.00	10.00	.00
86	76.47	1.00	m	81.00	1.00	12.00	1.00

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Age	Age Code ≤ 65/ >65yr	Sex	Preoperative CRP mg/l	CRP Code (preop) < 10/ > 10mg/l	Postoperative CRP mg/l	CRP Code (postop) < 10/ > 10mg/l
87	57.32	.00	m	5.00	.00	5.00	.00
88	91.10	1.00	m	5.00	.00	7.00	.00
89	61.54	.00	m	66.00	1.00	15.00	1.00
90	89.10	1.00	m	5.00	.00	14.00	1.00
91	74.10	1.00	m	7.00	.00	6.00	.00
92	50.68	.00	m	5.00	.00	21.00	1.00
93	86.82	1.00	f	94.00	1.00	113.00	1.00
94	71.19	1.00	m	34.00	1.00	202.00	1.00
95	83.36	1.00	m	5.00	.00	20.00	1.00
96	86.47	1.00	m	5.00	.00	5.00	.00
97	87.28	1.00	f	8.00	.00	5.00	.00
98	57.20	.00	f	19.00	1.00	8.00	.00
99	57.38	.00	m	50.00	1.00		
100	79.65	1.00	f	5.00	.00		
101	62.85	.00	f	5.00	.00	9.00	.00
102	64.73	.00	f	46.00	1.00		
103	92.02	1.00	f	19.00	1.00	57.00	1.00

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Stage & Grade	Stage Code superficial/ invasive	Grade Code G1/G2/G3	Recurrence	Progression	Treatment Code	Survival in Months
1	T1 G1	.00	1.00	1.00	1.00	2.00	96.87
2	TaG1	.00	1.00	.00	.00	.00	47.90
3	T2G3	1.00	3.00	.00	.00	.00	5.37
4	T2 G3	1.00	3.00	.00	.00	.00	.10
5	T1G3+CIS	1.00	3.00	.00	.00	.00	3.20
6	T1G2	.00	2.00	.00	.00	.00	12.10
7	T1G1	.00	1.00	1.00	1.00	3.00	46.67
8	T2G2	1.00	2.00	1.00	.00	1.00	26.23
9	T2G3	1.00	3.00	.00	.00	.00	1.23
10	TaG1	.00	1.00	1.00	.00	.00	5.17
11	T2G3	1.00	3.00	.00	.00	.00	10.20
12	T2G3+CIS	1.00	3.00	.00	.00	.00	141.30
13	TaG1	.00	1.00	1.00	.00	.00	23.80
14	TaG1	.00	1.00	.00	.00	.00	138.30
15	TaG1	.00	1.00	.00	.00	.00	67.70
16	T2G3	1.00	3.00	.00	.00	.00	1.67
17	TaG2	.00	2.00	1.00	.00	.00	44.03

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Stage & Grade	Stage Code superficial/ invasive	Grade Code G1/G2/G3	Recurrence	Progression	Treatment Code	Survival in Months
18	TaG1	.00	1.00	1.00	1.00	.00	95.33
19	TaG1	.00	1.00	.00	.00	.00	78.23
20	T1G3	1.00	3.00	.00	.00	.00	2.50
21	T1G3	1.00	3.00	1.00	.00	.00	117.50
22	TaG2	.00	2.00	1.00	.00	.00	27.37
23	TaG2	.00	2.00	.00	.00	.00	30.10
24	TaG1	.00	1.00	.00	.00	.00	68.00
25	TaG2	.00	2.00	.00	.00	.00	2.00
26	T2G3	1.00	3.00	.00	.00	2.00	12.20
27	T2G3	1.00	3.00	.00	.00	.00	2.87
28	TaG1	.00	1.00	1.00	1.00	.00	69.30
29	TaG2	.00	2.00	1.00	.00	2.00	80.70
30	TaG2	.00	2.00	.00	.00	.00	1.83
31	T2G3	1.00	3.00	1.00	1.00	1.00	33.20
32	T1G3	1.00	3.00	1.00	.00	2.00	18.87
33	T1G2	.00	2.00	.00	.00	.00	32.43
34	TaG2	.00	2.00	1.00	.00	3.00	76.17

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Stage & Grade	Stage Code superficial/ invasive	Grade Code G1/G2/G3	Recurrence	Progression	Treatment Code	Survival in Months
35	TaG1	.00	1.00	.00	.00	.00	14.47
36	T2GX	1.00	2.00	.00	.00	.00	15.43
37	TaG1	.00	1.00	.00	.00	.00	92.63
38	TaG1	.00	1.00	1.00	.00	3.00	91.57
39	T1G2	.00	2.00	1.00	.00	.00	90.50
40	TaG1	.00	1.00	1.00	.00	.00	38.67
41	TaG1	.00	1.00	1.00	1.00	.00	54.07
42	TaG1	.00	1.00	1.00	.00	.00	87.70
43	Tag2	.00	2.00	1.00	1.00	.00	24.10
44	T1G3	1.00	3.00	1.00	1.00	2.00	50.67
45	T2G2	1.00	2.00	1.00	.00	2.00	41.87
46	T3G3	1.00	3.00	.00	.00	.00	1.67
47	TaG1	.00	1.00	.00	.00	.00	74.10
48	T2G2	1.00	2.00	.00	.00	.00	6.23
49	T1G3	1.00	3.00	.00	.00	3.00	73.73
50	TaG1	.00	1.00	.00	.00	.00	72.97
51	TaG2	.00	2.00	.00	.00	.00	71.27

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Stage & Grade	Stage Code superficial/ invasive	Grade Code G1/G2/G3	Recurrence	Progression	Treatment Code	Survival in Months
52	TaG1	.00	1.00	.00	.00	.00	70.17
53	T1G3	1.00	3.00	.00	.00	2.00	69.47
54	T1G3	1.00	3.00	1.00	.00	.00	23.60
55	TaG2	.00	2.00	.00	.00	.00	69.27
56	CIS	.00	3.00	.00	.00	3.00	69.00
57	T2G2	1.00	2.00	1.00	1.00	2.00	32.33
58	T2G3	1.00	3.00	.00	.00	.00	15.70
59	T2G3	1.00	3.00	1.00	.00	1.00	8.60
60	TaG2	.00	2.00	.00	.00	.00	40.93
61	TaG2	.00	2.00	.00	.00	.00	66.37
62	T1G2	.00	2.00	1.00	.00	.00	32.63
63	TaG1	.00	1.00	1.00	.00	.00	65.03
64	TaG1	.00	1.00	1.00	.00	.00	32.60
65	T2G3	1.00	3.00	.00	.00	.00	1.60
66	T1G2	.00	2.00	.00	.00	.00	64.33
67	T2G3	1.00	3.00	.00	.00	.00	32.73
68	T1G3	1.00	3.00	.00	.00	.00	1.77

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Stage & Grade	Stage Code superficial/ invasive	Grade Code G1/G2/G3	Recurrence	Progression	Treatment Code	Survival in Months
69	T2G3	1.00	3.00	1.00	.00	.00	14.93
70	T2G2	1.00	2.00	.00	.00	.00	1.60
71	T2G3	1.00	3.00	.00	.00	.00	4.80
72	T1G3	1.00	3.00	1.00	.00	.00	59.50
73	T1G2	.00	2.00	1.00	.00	.00	58.20
74	TaG2	.00	2.00	1.00	1.00	.00	58.03
75	T2G3	1.00	3.00	1.00	.00	1.00	56.17
76	TaG2	.00	2.00	.00	.00	.00	56.10
77	TaG2	.00	2.00	.00	.00	.00	55.93
78	TaG1	.00	1.00	.00	.00	.00	55.47
79	TaG1	.00	1.00	.00	.00	.00	54.37
80	T2G3	1.00	3.00	1.00	.00	2.00	54.00
81	TaG1	.00	1.00	.00	.00	.00	30.50
82	TaG2	.00	2.00	.00	.00	.00	52.90
83	TaG3	.00	3.00	1.00	1.00	1.00	52.60
84	TaG2	.00	2.00	1.00	.00	.00	52.43
85	TaG2	.00	2.00	.00	.00	.00	52.40
86	TaG2	.00	2.00	.00	.00	.00	52.00

Appendix 2.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 4

Patient No.	Stage & Grade	Stage Code superficial/ invasive	Grade Code G1/G2/G3	Recurrence	Progression	Treatment Code	Survival in Months
87	TaG2	.00	2.00	1.00	.00	.00	52.00
88	T2G3	1.00	3.00	.00	.00	.00	1.67
89	TaG2	.00	2.00	1.00	.00	.00	51.93
90	TAG2	.00	2.00	1.00	.00	.00	6.60
91	T2G3	1.00	3.00	1.00	1.00	1.00	33.83
92	TaG2	.00	2.00	.00	.00	.00	13.07
93	T2G3	1.00	3.00	.00	.00	.00	2.90
94	T1G3	1.00	3.00	.00	.00	.00	3.30
95	T2G3	1.00	3.00	.00	.00	.00	2.00
96	TaG1	.00	1.00	1.00	.00	3.00	49.20
97	T2G3	1.00	3.00	.00	.00	.00	24.47
98	TaG2	.00	2.00	.00	.00	.00	48.77
99	TaG3	.00	3.00	.00	.00	.00	1.97
100	TaG2	.00	2.00	.00	.00	.00	46.80
101	T1G2	.00	2.00	1.00	.00	.00	35.50
102	T2G3	1.00	3.00	1.00	.00	1.00	19.27
103	T2G3	1.00	3.00	.00	.00	.00	14.07

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	Follow-up Code	Cause of Death	CD-4	CD-4 Tertiles	CD-8	CD-8 Tertiles	CD-4+8	CD-4+8 Tertiles
1	2.00	Myelodysplastic Syndrome	.96	.00	.16	.00	1.12	.00
2	2.00	MI	.40	.00	3.20	1.00	3.60	.00
3	1.00	Bladder Cancer	1.70	1.00	1.80	1.00	3.50	1.00
4	1.00	bladder Cancer	1.70	1.00	7.00	2.00	8.70	1.00
5	1.00	Bladder Cancer	3.50	1.00	5.20	2.00	8.70	1.00
6	1.00	Bladder Cancer	2.30	1.00	.33	.00	2.63	.00
7	1.00	Bladder Cancer	7.00	2.00	1.70	1.00	8.70	.00
8	2.00	Peritonitis	.50	.00	.73	.00	1.23	1.00
9	2.00	Asthma	.10	.00	1.80	1.00	1.90	1.00
10	2.00	MI	.00	.00	3.00	1.00	3.00	.00
11	1.00	Bladder Cancer	1.30	.00	6.70	2.00	8.00	1.00
12	.00		1.50	.00	6.10	2.00	7.60	1.00
13	2.00	COAD	.20	.00	1.40	.00	1.60	.00
14	.00		.20	.00	3.00	1.00	3.20	.00
15	2.00	Amputation / Dementia	3.70	1.00	6.10	2.00	9.80	.00
16	1.00	Bladder Cancer	1.63	.00	1.53	1.00	3.16	1.00
17	1.00	Bladder ca	2.33	1.00	1.86	1.00	4.19	.00

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	Follow-up Code	Cause of Death	CD-4	CD-4 Tertiles	CD-8	CD-8 Tertiles	CD-4+8	CD-4+8 Tertiles
18	2.00	PE	3.13	1.00	.73	.00	3.86	.00
19	2.00	Pneumonia	.40	.00	.33	.00	.73	.00
20	1.00	Bladder Cancer	.96	.00	1.56	1.00	2.52	1.00
21	.00		2.86	1.00	1.00	.00	3.86	1.00
22	1.00	Bladder Cancer	.40	.00	2.20	1.00	2.60	.00
23	2.00	Pneumonia	1.20	.00	3.30	1.00	4.50	.00
24	1.00	Bladder Cancer	.10	.00	.60	.00	.70	.00
25	1.00	Bladder Cancer	.40	.00	5.70	2.00	6.10	.00
26	2.00	CVA	1.20	.00	.80	.00	2.00	1.00
27	1.00	Bladder Cancer	2.80	1.00	3.70	2.00	6.50	1.00
28	1.00	Bladder Cancer	.10	.00	.50	.00	.60	.00
29	2.00	Lung Cancer	1.60	.00	1.23	.00	2.83	.00
30	1.00	Bladder Cancer	8.86	2.00	4.70	2.00	13.56	.00
31	1.00	Bladder Cancer	.40	.00	1.60	1.00	2.00	1.00
32	1.00	Bladder Cancer	.80	.00	4.10	2.00	4.90	1.00
33	2.00	COAD	.93	.00	1.80	1.00	2.73	.00
34	1.00	Bladder Cancer	5.70	2.00	.50	.00	6.20	.00

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	Follow-up Code	Cause of Death	CD-4	CD-4 Tertiles	CD-8	CD-8 Tertiles	CD-4+8	CD-4+8 Tertiles
35	2.00	Leukaemia	2.10	1.00	1.43	.00	3.53	.00
36	1.00	Bladder Cancer	3.00	1.00	1.00	.00	4.00	1.00
37	.00		1.50	.00	1.00	.00	2.50	.00
38	.00		1.70	1.00	3.60	2.00	5.30	.00
39	.00		.60	.00	3.60	1.00	4.20	.00
40	2.00	IHD	2.60	1.00	2.40	1.00	5.00	.00
41	1.00	Bladder Cancer	3.40	1.00	1.30	.00	4.70	.00
42	.00		1.60	.00	1.40	.00	3.00	.00
43	2.00	COAD	3.90	1.00	4.60	2.00	8.50	.00
44	1.00	Bladder Cancer	5.30	2.00	5.20	2.00	10.50	1.00
45	1.00	Bladder Cancer	4.00	2.00	3.90	2.00	7.90	1.00
46	1.00	Bladder Cancer	4.90	2.00	7.80	2.00	12.70	1.00
47	.00		3.00	1.00	1.10	.00	4.10	.00
48	1.00	Bladder Cancer	4.00	2.00	1.20	.00	5.20	1.00
49	.00		1.70	1.00	1.20	.00	2.90	1.00
50	.00		.80	.00	4.00	2.00	4.80	.00
51	.00		3.50	1.00	2.50	1.00	6.00	.00

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	Follow-up Code	Cause of Death	CD-4	CD-4 Tertiles	CD-8	CD-8 Tertiles	CD-4+8	CD-4+8 Tertiles
52	.00		1.60	.00	1.00	.00	2.60	.00
53	.00		2.00	1.00	1.50	1.00	3.50	1.00
54	1.00	Bladder Cancer	8.70	2.00	5.60	2.00	14.30	1.00
55	.00		1.50	.00	3.00	1.00	4.50	.00
56	.00		7.30	2.00	9.60	2.00	16.90	.00
57	1.00	Bladder Cancer	4.50	2.00	2.20	1.00	6.70	1.00
58	1.00	Bladder Cancer	1.60	.00	1.10	.00	2.70	1.00
59	1.00	Bladder Cancer	5.40	2.00	4.40	2.00	9.80	1.00
60	2.00	COAD	1.50	.00	.90	.00	2.40	.00
61	.00		1.70	.00	.70	.00	2.40	.00
62	1.00	Bladder ca	8.90	2.00	3.90	2.00	12.80	.00
63	.00		4.10	2.00	1.60	1.00	5.70	.00
64	2.00	Peritonitis	2.80	1.00	1.70	1.00	4.50	.00
65	1.00	Bladder Cancer	10.90	2.00	7.30	2.00	18.20	1.00
66	.00		6.00	2.00	3.10	1.00	9.10	.00
67	2.00	Prostatic Cancer	1.90	1.00	1.20	.00	3.10	1.00
68	1.00	Bladder Cancer	7.20	2.00	4.30	2.00	11.50	1.00

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	Follow-up Code	Cause of Death	CD-4	CD-4 Tertiles	CD-8	CD-8 Tertiles	CD-4+8	CD-4+8 Tertiles
69	1.00	Bladder Cancer	3.70	1.00	1.90	1.00	5.60	1.00
70	1.00	Bladder Cancer	6.60	2.00	3.10	1.00	9.70	1.00
71	1.00	Bladder Cancer	11.10	2.00	10.20	2.00	21.30	1.00
72	.00		12.30	2.00	7.10	2.00	19.40	1.00
73	.00		3.60	1.00	5.40	2.00	9.00	.00
74	.00		5.30	2.00	2.40	1.00	7.70	.00
75	.00		6.20	2.00	6.60	2.00	12.80	1.00
76	.00		5.20	2.00	2.00	1.00	7.20	.00
77	.00		4.00	1.00	1.20	.00	5.20	.00
78	.00		4.60	2.00	4.60	2.00	9.20	.00
79	.00		1.90	1.00	.60	.00	2.50	.00
80	.00		4.60	2.00	4.00	2.00	8.60	1.00
81	2.00	CVA	1.20	.00	1.20	.00	2.40	.00
82	.00		4.40	2.00	1.40	.00	5.80	.00
83	.00		.30	.00	2.30	1.00	2.60	.00
84	.00		3.30	1.00	2.20	1.00	5.50	.00
85	.00		3.30	1.00	3.60	2.00	6.90	.00
86	.00		2.70	1.00	1.40	.00	4.10	.00

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	Follow-up Code	Cause of Death	CD-4	CD-4 Tertiles	CD-8	CD-8 Tertiles	CD-4+8	CD-4+8 Tertiles
87	.00		3.50	1.00	3.70	2.00	7.20	.00
88	1.00	Bladder Cancer	4.20	2.00	1.80	1.00	6.00	1.00
89	.00		4.40	2.00	1.90	1.00	6.30	.00
90	2.00	Tongue Ca	1.50	.00	1.00	.00	2.50	.00
91	1.00	Bladder Cancer	2.40	1.00	3.10	1.00	5.50	1.00
92	2.00	MI	5.60	2.00	3.70	2.00	9.30	.00
93	1.00	Bladder Cancer	2.00	1.00	1.90	1.00	3.90	1.00
94	1.00	Bladder Cancer	1.80	1.00	2.90	1.00	4.70	1.00
95	1.00	Bladder Cancer	9.50	2.00	6.60	2.00	16.10	1.00
96	.00		2.00	1.00	1.30	.00	3.30	.00
97	1.00	Bladder Cancer	9.20	2.00	7.50	2.00	16.70	1.00
98	.00		1.80	1.00	1.40	.00	3.20	.00
99	2.00	Intestinal Cancer	6.60	2.00	3.50	1.00	10.10	.00
100	.00		1.00	.00	1.50	.00	2.50	.00
101	2.00	Sepsis	6.20	2.00	4.60	2.00	10.80	.00
102	1.00	Bladder Cancer	8.50	2.00	3.90	2.00	12.40	1.00
103	1.00	Bladder Cancer	5.70	2.00	1.40	.00	7.10	1.00

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	KI-67	KI-67 Tertiles	COX-2	COX-2 Tertiles	CD 68	CD 68 Tertiles	CD 34	CD 34 Tertiles
1	19.00	.00	100	.00	49	1	45	1
2	20.00	.00	160	.00	46	0	46	1
3	43.00	1.00	120	.00	66	2	79	2
4	67.00	2.00	300	2.00	71	2	80	2
5	58.00	2.00	280	2.00	62	1	77	2
6	53.00	2.00	200	1.00	51	1	71	2
7	67.00	2.00	200	1.00	50	1	84	2
8	46.00	2.00	280	2.00	66	2	94	2
9	64.00	2.00	100	.00	50	1	59	1
10	4.00	.00	140	.00	29	0	48	1
11	39.00	1.00	270	2.00	74	2	78	2
12	24.00	.00	240	2.00	80	2	57	1
13	30.00	1.00	180	1.00	65	2	82	2
14	21.00	.00	110	.00	40	0	25	0
15	21.00	.00	170	.00	41	0	57	1
16	34.00	1.00	110	.00	77	2	73	2
17	37.00	1.00	130	.00	55	1	43	1

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	KI-67	KI-67 Tertiles	COX-2	COX-2 Tertiles	CD 68	CD 68 Tertiles	CD 34	CD 34 Tertiles
18	29.00	1.00	130	.00	62	1	28	0
19	19.00	.00	110	.00	31	0	31	0
20	39.00	1.00	280	2.00	69	2	71	2
21	50.00	2.00	270	2.00	59	1	78	2
22	57.00	2.00	190	1.00	49	1	73	2
23	4.00	.00	150	.00	49	1	48	1
24	2.00	.00	180	1.00	76	2	86	2
25	16.00	.00	180	1.00	49	1	81	2
26	33.00	1.00	270	2.00	65	2	80	2
27	30.00	1.00	280	2.00	66	2	77	2
28	21.00	.00	240	2.00	49	1	81	2
29	19.00	.00	110	.00	58	1	41	0
30	66.00	2.00	270	2.00	66	2	81	2
31	17.00	.00	230	1.00	77	2	74	2
32	32.00	1.00	280	2.00	57	1	79	2
33	14.00	.00	150	.00	43	0	20	0
34	16.00	.00	180	1.00	47	1	49	1

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	KI-67	KI-67 Tertiles	COX-2	COX-2 Tertiles	CD 68	CD 68 Tertiles	CD 34	CD 34 Tertiles
35	16.00	.00	200	1.00	52	1	49	1
36	4.00	.00	260	2.00	56	1	42	0
37	23.00	.00	110	.00	44	0	24	0
38	40.00	1.00	180	.00	73	2	21	0
39	31.00	1.00	190	1.00	41	0	52	1
40	26.00	1.00	190	1.00	60	1	71	2
41	35.00	1.00	190	1.00	54	1	79	2
42	36.00	1.00	180	.00	19	0	22	0
43	24.00	.00	150	.00	40	0	42	0
44	54.00	2.00	290	2.00	54	1	98	2
45	24.00	.00	160	.00	54	1	50	1
46	61.00	2.00	300	2.00	98	2	52	1
47	29.00	1.00	180	.00	30	0	22	0
48	49.00	2.00	280	2.00	68	2	53	1
49	32.00	1.00	220	1.00	49	1	54	1
50	17.00	.00	100	.00	25	0	46	1
51	36.00	1.00	180	.00	36	0	60	1

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	KI-67	KI-67 Tertiles	COX-2	COX-2 Tertiles	CD 68	CD 68 Tertiles	CD 34	CD 34 Tertiles
52	5.00	.00	120	.00	21	0	31	0
53	24.00	.00	230	1.00	47	1	45	1
54	67.00	2.00	300	2.00	55	1	71	2
55	25.00	.00	140	.00	18	0	67	1
56	33.00	1.00	210	1.00	57	1	25	0
57	36.00	1.00	270	2.00	69	2	74	2
58	62.00	2.00	240	2.00	62	1	47	1
59	67.00	2.00	240	2.00	66	2	76	2
60	13.00	.00	130	.00	28	0	50	1
61	26.00	1.00	190	1.00	47	1	27	0
62	43.00	1.00	200	1.00	30	0	49	1
63	60.00	2.00	190	1.00	72	2	68	2
64	19.00	.00	190	1.00	41	0	46	1
65	72.00	2.00	280	2.00	84	2	73	2
66	23.00	.00	130	.00	44	0	21	0
67	30.00	1.00	250	2.00	98	2	70	2
68	65.00	2.00	250	2.00	72	2	81	2

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	KI-67	KI-67 Tertiles	COX-2	COX-2 Tertiles	CD 68	CD 68 Tertiles	CD 34	CD 34 Tertiles
69	76.00	2.00	290	2.00	67	2	77	2
70	51.00	2.00	200	1.00	81	2	49	1
71	71.00	2.00	210	1.00	84	2	32	0
72	54.00	2.00	240	2.00	61	1	74	2
73	44.00	2.00	200	1.00	62	1	49	1
74	43.00	1.00	180	1.00	32	0	33	0
75	46.00	2.00	220	1.00	73	2	58	1
76	18.00	.00	180	.00	43	0	41	0
77	31.00	1.00	250	2.00	59	1	23	0
78	4.00	.00	190	1.00	42	0	29	0
79	14.00	.00	120	.00	34	0	49	1
80	38.00	1.00	220	1.00	70	2	45	1
81	7.00	.00	190	1.00	19	0	50	1
82	21.00	.00	110	.00	41	0	39	0
83	34.00	1.00	210	1.00	40	0	24	0
84	7.00	.00	120	.00	45	0	42	1
85	58.00	2.00	220	1.00	42	0	39	0
86	51.00	2.00	180	.00	42	0	32	0

Appendix 2.2 Immunohistochemical expression in Transitional Cell Carcinoma of the Urinary Bladder for Patients Described in Chapter 4

Patient No.	KI-67	KI-67 Tertiles	COX-2	COX-2 Tertiles	CD 68	CD 68 Tertiles	CD 34	CD 34 Tertiles
87	32.00	1.00	200	1.00	39	0	31	0
88	62.00	2.00	270	2.00	51	1	52	1
89	53.00	2.00	250	2.00	57	1	56	1
90	30.00	1.00	200	1.00	64	1	24	0
91	37.00	1.00	270	2.00	73	2	53	1
92	34.00	1.00	200	1.00	42	0	32	0
93	33.00	1.00	270	2.00	79	2	31	0
94	57.00	2.00	280	2.00	68	2	79	2
95	80.00	2.00	220	1.00	68	2	33	0
96	35.00	1.00	180	.00	42	0	21	0
97	62.00	2.00	230	1.00	76	2	47	1
98	4.00	.00	150	.00	69	2	26	0
99	76.00	2.00	220	1.00	55	1	77	2
100	9.00	.00	130	.00	33	0	35	0
101	45.00	2.00	250	2.00	40	0	25	0
102	71.00	2.00	280	2.00	67	2	79	2
103	44.00	1.00	260	2.00	73	2	28	0

APPENDIX 3: DATA FROM THE PROSPECTIVE STUDY FOR PATIENTS WITH TRANSITIONAL CELL CARCINOMA OF THE URINARY BLADDER DESCRIBED IN CHAPTER 5

There follows the raw data collected for the prospective study for patients with transitional cell carcinoma of the urinary bladder which is discussed in chapter 5.

Abbreviations Used in Tables

Treatment Code:

0 = transurethral resection of bladder tumour + single intravesical mitomycin

1 = Cystectomy

2 = Radical radiotherapy

3 = Intravesical BCG

IFNG: Interferon gamma

GM-CSF: Granulocyte / macrophage colony-stimulating factor

TNFA: Tumour necrosis factor alpha

RANTES: Regulated on Activation, Normal T Expressed and Secreted

MIG: Monokine induced by interferon gamma

MIP: Monocyte inflammatory protein

MCP: Monocyte chemo-attractant protein

Appendix 3.1 Clinicopathological Characteristics for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 5

Patient's No.	Age	Age Code ≤ 65 / > 65 yr	Sex	Frank Hacmaturia	Irritative Symptoms	Preoperative CRP mg/l	CRP Code (preop) < 10/ > 10mg/l
1	79.00	1.00	m	.00	.00	6.00	.00
2	63.00	.00	f	.00	.00	6.00	.00
3	73.00	1.00	m	1.00	.00	6.00	.00
4	64.00	.00	m	1.00	.00	16.00	1.00
5	89.00	1.00	m	1.00	.00	9.00	.00
6	77.00	1.00	m	1.00	.00	7.00	.00
7	60.00	.00	m	1.00	.00	26.00	1.00
8	60.00	.00	m	1.00	1.00	6.00	.00
9	81.00	1.00	m	1.00	.00	36.00	1.00
10	75.00	1.00	m	1.00	.00	11.00	1.00
11	78.00	1.00	f	1.00	.00	44.00	1.00
12	84.00	1.00	m	1.00	.00	6.00	.00
13	57.00	.00	f	1.00	.00	60.00	1.00
14	78.00	1.00	m	1.00	.00	12.00	1.00
15	81.00	1.00	m	1.00	.00	6.00	.00
16	69.00	1.00	f	1.00	.00	9.00	.00
17	53.00	.00	m	1.00	.00	6.00	.00

Appendix 3.1 Clinicopathological characteristics for patients with transitional cell carcinoma of the urinary bladder Described in chapter 5

Patient's No.	Age	Age Code ≤ 65 / > 65 yr	Sex	Frank Haematuria	Irritative Symptoms	Preoperative CRP mg/l	CRP Code (preop) < 10/ > 10mg/l
18	64.00	.00	m	1.00	.00	6.00	.00
19	65.00	.00	f	.00	.00	6.00	.00
20	63.00	.00	m	1.00	.00	6.00	.00
21	71.00	1.00	m	.00	1.00	7.00	.00
22	57.00	.00	m	1.00	.00	9.00	.00
23	70.00	1.00	f	.00	1.00	6.00	.00
24	70.00	1.00	m	1.00	.00	22.00	1.00
25	70.00	1.00	m	1.00	.00	6.00	.00
26	78.00	1.00	m	.00	.00	6.00	.00
27	70.00	1.00	f	1.00	.00	6.00	.00
28	64.00	.00	m	1.00	.00	11.00	1.00
29	78.00	1.00	f	1.00	.00	6.00	.00
30	80.00	1.00	m	1.00	.00	14.00	1.00
31	57.00	.00	f	1.00	.00	14.00	1.00
32	70.00	1.00	m	1.00	.00	6.00	.00
33	66.00	1.00	m	.00	.00	13.00	1.00
34	74.00	1.00	f	1.00	1.00	21.00	1.00

Appendix 3.1 Clinicopathological characteristics for patients with transitional cell carcinoma of the urinary bladder Described in chapter 5

Patient's No.	Age	Age Code ≤ 65 / > 65 yr	Sex	Frank Haematuria	Irritative Symptoms	Preoperative CRP mg/l	CRP Code (preop) < 10/ > 10mg/l
35	71.00	1.00	f	1.00	.00	9.00	.00
36	54.00	.00	f	.00	1.00	12.00	1.00
37	64.00	.00	M	.00	.00	10.00	.00
38	54.00	.00	m	1.00	.00	9.00	.00
39	75.00	1.00	m	1.00	.00	10.00	.00
40	80.00	1.00	m	1.00	.00	6.00	.00
41	69.00	1.00	m	1.00	.00	15.00	1.00
42	81.00	1.00	f	1.00	.00	8.00	.00
43	73.00	1.00	m	1.00	.00	10.00	.00
44	80.00	1.00	m	1.00	1.00	83.00	1.00
45	73.00	1.00	m	1.00	.00	7.00	.00
46	72.00	1.00	m	1.00	.00	6.00	.00
47	68.00	1.00	m	1.00	.00	6.00	.00

Appendix 3.1 Clinicopathological characteristics for patients with transitional cell carcinoma of the urinary bladder Described in chapter 5

Patient's No.	Postoperative CRP mg/l	CRP Code (postop) < 10/ > 10mg/l	Stage	Stage Code superficial/ invasive	Grade	Grade Code	Treatment Code	Recurrence
1			Ta	.00	G2	2.00	.00	.00
2	6.00	.00	Ta	.00	G2	2.00	.00	.00
3	9.00	.00	Ta	.00	G3	3.00	.00	1.00
4	9.00	.00	Ta	.00	G2	2.00	.00	1.00
5			T2	1.00	G3	3.00	.00	.00
6	6.00	.00	Ta	.00	G2	2.00	.00	.00
7	37.00	1.00	T1	1.00	G3	3.00	3.00	1.00
8	6.00	.00	Ta	.00	G2	2.00	.00	1.00
9	14.00	1.00	T1	1.00	G3	3.00	.00	1.00
10	12.00	1.00	Ta	.00	G2	2.00	.00	.00
11	6.00	.00	Ta	.00	G1	1.00	.00	.00
12			Ta	.00	G1	1.00	.00	.00
13			T2	1.00	G2	2.00	.00	.00
14			T2	1.00	G3	3.00	2.00	1.00
15			T2	1.00	G3	3.00	3.00	1.00
16	12.00	1.00	Ta	.00	G2	2.00	.00	.00
17	6.00	.00	Ta	.00	G2	2.00	.00	.00

Appendix 3.1 Clinicopathological characteristics for patients with transitional cell carcinoma of the urinary bladder Described in chapter 5

Patient's No.	Postoperative CRP mg/l	CRP Code (postop) < 10/ > 10mg/l	Stage	Stage Code superficial/ invasive	Grade	Grade Code	Treatment Code	Recurrence
18			Ta	.00	G1	1.00	.00	.00
19	6.00	.00	Ta	.00	G2	2.00	.00	.00
20	7.00	.00	T1	.00	G2	2.00	1.00	1.00
21			T3	1.00	G3	3.00	1.00	1.00
22			Ta	.00	G1	1.00	.00	.00
23			T2	1.00	G3	3.00	.00	.00
24	6.00	.00	Ta	.00	G1	1.00	3.00	1.00
25	8.00	.00	T1	1.00	G3	3.00	1.00	1.00
26	15.00	1.00	Ta	.00	G2	2.00	.00	1.00
27	6.00	.00	T2	1.00	G3	3.00	2.00	1.00
28	6.00	.00	T1	.00	G2	2.00	3.00	1.00
29	14.00	1.00	Ta	.00	G3	3.00	.00	.00
30	204.00	1.00	Ta	.00	G2	2.00	.00	.00
31	6.00	.00	T3	1.00	G3	3.00	1.00	1.00
32	8.00	.00	Ta	.00	G3	3.00	3.00	.00
33			Ta	.00	G2	2.00	.00	.00
34	200.00	1.00	T2	1.00	G3	3.00	1.00	1.00

Appendix 3.1 Clinicopathological characteristics for patients with transitional cell carcinoma of the urinary bladder Described in chapter 5

Patient's No.	Postoperative CRP mg/l	CRP Code (postop) < 10/ > 10mg/l	Stage	Stage Code superficial/ invasive	Grade	Grade Code	Treatment Code	Recurrence
35	6.00	.00	Ta	.00	G3	3.00	3.00	.00
36			Ta	.00	G1	1.00	.00	.00
37	10.00	.00	Ta	.00	G3	3.00	3.00	1.00
38	6.00	.00	Ta	.00	G2	2.00	.00	.00
39	6.00	.00	T1	1.00	G3	3.00	.00	.00
40	6.00	.00	Ta	.00	G2	2.00	.00	.00
41			Ta	.00	G2	2.00	.00	.00
42	9.00	.00	T1	.00	G2	2.00	.00	.00
43			Ta	.00	G1	1.00	.00	.00
44			T2	1.00	G2	2.00	.00	1.00
45	6.00	.00	Ta	.00	G3	3.00	.00	1.00
46	6.00	.00	T3	1.00	G3	3.00	1.00	1.00
47	29.00	1.00	T3	1.00	G3	3.00	1.00	1.00

Appendix 3.2 Cytokines Profile for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 5

Pati ent's No.	IL-1B	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
1	.00	2.00	.00	2.00	10.00	.00	1.00	5.00
2	197.00	53.00	2.00	2.00	12.00	.00	2.00	3.00
3	.00	.00	.00	53.00	2586.00	590.00	1.00	1216.00
4	1422.00	1469.00	166.00	4.00	173.00	172.00	2.00	19.00
5	51.00	.00	3.00	.00	132.00	52.00	10.00	43.00
6	118.00	3.00	6.00	.00	14.00	.00	6.00	10.00
7	.00	.00	1.00	1.00	12.00	.00	1.00	3.00
8	.00	.00	.00	3.00	.00	.00	1.00	1.00
9	6.00	5.00	.00	2.00	1.00	.00	1.00	4.00
10	77.00	4.00	2.00	2.00	5.00	27.00	2.00	.00
11	22.00	4.00	1.00	2.00	3.00	7.00	3.00	4.00
12	.00	.00	.00	2.00	.00	.00	1.00	4.00
13	108.00	4.00	1.00	2.00	21.00	.00	1.00	8.00
14	104.00	17.00	.00	2.00	2.00	4.00	1.00	2.00
15	.00	3.00	.00	2.00	1.00	.00	2.00	.00
16	.00	.00	.00	2.00	6.00	.00	1.00	4.00
17	84.00	7.00	1.00	2.00	1.00	.00	1.00	.00

Appendix 3.2 Cytokines Profile for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 5

Patient's No.	IL-1B	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
18	42.00	7.00	.00	1.00	1.00	.00	1.00	3.00
19	.00	.00	.00	2.00	1.00	.00	1.00	1.00
20	1465.00	1960.00	201.00	26.00	65.00	62.00	3.00	181.00
21	35.00	.00	.00	2.00	6.00	7.00	1.00	9.00
22	.00	.00	.00	2.00	2.00	.00	1.00	1.00
23	500.00	21.00	1.00	2.00	14.00	6.00	5.00	.00
24	110.00	11.00	2.00	1.00	6.00	.00	2.00	.00
25	329.00	40.00	1.00	2.00	.00	.00	1.00	1.00
26	.00	12.00	.00	2.00	80.00	17.00	3.00	.00
27	23.00	9.00	2.00	2.00	3.00	18.00	2.00	1.00
28	100.00	.00	.00	3.00	7.00	.00	7.00	12.00
29	24.00	.00	.00	2.00	6.00	.00	1.00	11.00
30	.00	4.00	9.00	.00	33.00	78.00	46.00	.00
31	.00	1.00	1.00	2.00	5.00	4.00	39.00	.00
32	128.00	9.00	1.00	3.00	4.00	10.00	1.00	5.00
33	18.00	7.00	.00	1.00	3.00	.00	2.00	1.00
34	.00	.00	.00	2.00	4.00	.00	2.00	.00

Appendix 3.2 Cytokines Profile for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 5

Patient's No.	IL-1B	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	IL-10
35	.00	.00	.00	1.00	6.00	.00	1.00	1.00
36	.00	.00	.00	2.00	3.00	.00	1.00	5.00
37	39.00	6.00	1.00	2.00	1.00	.00	1.00	2.00
38	.00	1.00	.00	2.00	2.00	.00	1.00	9.00
39	.00	1.00	1.00	2.00	2.00	7.00	1.00	2.00
40	.00	.00	.00	4.00	3.00	.00	18.00	1.00
41	47.00	2.00	1.00	2.00	6.00	5.00	1.00	3.00
42	107.00	4.00	2.00	3.00	5.00	8.00	198.00	1.00
43	131.00	24.00	.00	2.00	2.00	4.00	1.00	.00
44	81.00	2.00	1.00	2.00	42.00	11.00	2.00	10.00
45	121.00	17.00	1.00	2.00	1.00	4.00	1.00	5.00
46	64.00	14.00	.00	2.00	5.00	2.00	1.00	1.00
47	40.00	5.00	3.00	.00	14.00	71.00	5.00	8.00

Appendix 3.2 Cytokines Profile for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 5

Patient's No.	IL-12	IL-13	IL-15	GM-CSF	IFNG	TNFA
1	63.00	.00	5.00	.00	.00	.00
2	63.00	.00	18.00	4.00	.00	8.00
3	31.00	75.00	4.00	4132.00	.00	6.00
4	760.00	33.00	828.00	106.00	.00	863.00
5	275.00	.00	21.00	307.00	3.00	5.00
6	177.00	.00	20.00	.00	.00	7.00
7	39.00	.00	2.00	.00	.00	1.00
8	38.00	.00	1.00	.00	.00	1.00
9	32.00	.00	5.00	.00	.00	.00
10	27.00	.00	12.00	.00	.00	1.00
11	96.00	.00	4.00	.00	.00	3.00
12	35.00	.00	1.00	.00	.00	1.00
13	23.00	.00	8.00	.00	.00	3.00
14	30.00	.00	9.00	.00	.00	4.00
15	62.00	.00	3.00	.00	.00	1.00
16	36.00	.00	.00	.00	.00	3.00
17	42.00	.00	3.00	.00	.00	1.00

Appendix 3.2 Cytokines Profile for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 5

Patient's No.	IL-12	IL-13	IL-15	GM-CSF	IFNG	TNFA
18	33.00	.00	3.00	.00	.00	.00
19	27.00	.00	1.00	.00	.00	2.00
20	742.00	2.00	613.00	272.00	.00	236.00
21	48.00	.00	2.00	.00	.00	3.00
22	34.00	.00	1.00	.00	.00	1.00
23	56.00	.00	15.00	.00	.00	51.00
24	47.00	.00	5.00	.00	.00	3.00
25	75.00	.00	6.00	.00	.00	4.00
26	49.00	.00	.00	.00	.00	4.00
27	44.00	.00	6.00	.00	1.00	2.00
28	66.00	.00	3.00	.00	.00	3.00
29	59.00	.00	1.00	.00	.00	2.00
30	222.00	.00	14.00	.00	17.00	25.00
31	163.00	.00	3.00	.00	1.00	3.00
32	50.00	46.00	9.00	.00	1.00	3.00
33	35.00	.00	4.00	.00	.00	1.00
34	18.00	2.00	1.00	.00	.00	.00

Appendix 3.2 Cytokines Profile for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 5

Patient's No.	IL-12	IL-13	IL-15	GM-CSF	IFNG	TNFA
35	30.00	.00	4.00	.00	.00	1.00
36	51.00	.00	1.00	4.00	.00	2.00
37	42.00	.00	2.00	.00	.00	4.00
38	56.00	.00	.00	.00	.00	3.00
39	59.00	.00	4.00	.00	.00	2.00
40	98.00	.00	.00	.00	.00	.00
41	37.00	1.00	9.00	.00	.00	3.00
42	62.00	.00	8.00	.00	2.00	4.00
43	32.00	.00	11.00	.00	.00	.00
44	78.00	.00	7.00	.00	.00	7.00
45	40.00	.00	6.00	25.00	.00	3.00
46	43.00	.00	5.00	.00	.00	1.00
47	101.00	.00	17.00	.00	2.00	8.00

Appendix 3.2 Cytokines Profile for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 5

Patient's No.	RANTES	EOTAXIN	IP-10	MIG	MIP-1A	MIP-1B	MCP-1
1	3406.00	37.00	147.00	75.00	36.00	11.00	79.00
2	21628.00	112.00	14.00	.00	48.00	50.00	151.00
3	45438.00	34.00	65.00	.00	63.00	65.00	143.00
4	1283.00	219.00	75.00	576.00	705.00	2044.00	691.00
5	11643.00	48.00	40.00	116.00	56.00	84.00	150.00
6	7690.00	140.00	21.00	119.00	45.00	73.00	196.00
7	4755.00	69.00	37.00	.00	10.00	120.00	227.00
8	.00	51.00	45.00	.00	18.00	18.00	149.00
9	48163.00	56.00	34.00	.00	30.00	69.00	103.00
10	10783.00	114.00	23.00	.00	36.00	63.00	315.00
11	54236.00	84.00	65.00	.00	37.00	91.00	137.00
12	9103.00	37.00	30.00	.00	6.00	.00	66.00
13	32192.00	30.00	70.00	.00	39.00	49.00	149.00
14	23454.00	28.00	43.00	.00	44.00	79.00	63.00
15	70102.00	53.00	34.00	.00	17.00	.00	94.00
16	9077.00	56.00	37.00	76.00	15.00	8.00	104.00
17	7742.00	29.00	7.00	.00	71.00	113.00	104.00

Appendix 3.2 Cytokines Profile for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 5

Patient's No.	RANTES	EOTAXIN	IP-10	MIG	MIP-1A	MIP-1B	MCP-1
18	19000.00	36.00	30.00	.00	23.00	12.00	96.00
19	132605.00	1.00	29.00	.00	10.00	.00	87.00
20	22283.00	130.00	118.00	361.00	126.00	488.00	1500.00
21	14117.00	28.00	79.00	.00	28.00	18.00	134.00
22	20062.00	81.00	37.00	.00	17.00	5.00	161.00
23	17572.00	95.00	204.00	13.00	132.00	180.00	165.00
24	11909.00	90.00	26.00	.00	39.00	38.00	170.00
25	18022.00	81.00	27.00	.00	75.00	209.00	96.00
26	24649.00	73.00	30.00	.00	22.00	142.00	96.00
27	32226.00	120.00	26.00	2.00	25.00	9.00	124.00
28	4828.00	70.00	48.00	.00	21.00	28.00	93.00
29	25941.00	49.00	36.00	.00	20.00	39.00	107.00
30	51037.00	150.00	80.00	53.00	50.00	77.00	137.00
31	539602.00	79.00	25.00	.00	18.00	29.00	117.00
32	3845.00	34.00	55.00	.00	31.00	53.00	117.00
33	21104.00	46.00	23.00	.00	23.00	20.00	137.00
34	2122.00	14.00	46.00	34.00	8.00	103.00	107.00

Appendix 3.2 Cytokines Profile for Patients with Transitional Cell Carcinoma of the Urinary Bladder Described in Chapter 5

Patient's No.	RANTES	EOTAXIN	IP-10	MIG	MIP-1A	MIP-1B	MCP-1
35	15381.00	70.00	33.00	.00	33.00	24.00	98.00
36	17644.00	30.00	24.00	.00	9.00	3.00	174.00
37	24148.00	38.00	33.00	.00	21.00	.00	120.00
38	44136.00	172.00	24.00	.00	36.00	35.00	360.00
39	14278.00	64.00	35.00	.00	28.00	58.00	98.00
40	16860.00	101.00	35.00	2.00	1.00	34.00	173.00
41	31535.00	54.00	73.00	.00	37.00	.00	100.00
42	131302.00	63.00	47.00	.00	38.00	21.00	137.00
43	2174.00	35.00	26.00	.00	51.00	41.00	173.00
44	2805.00	94.00	59.00	.00	37.00	117.00	245.00
45	9097.00	83.00	28.00	.00	54.00	84.00	95.00
46	23624.00	82.00	43.00	.00	34.00	70.00	115.00
47	11990.00	58.00	32.00	40.00	74.00	130.00	146.00