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Proliferation in the Rat Urinary Bladder in Response to Experimentally-induced Cystitis

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I proclaim that thesis was written by myself and all informations were used from the presented references.

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List of abbreviations

ATP	adenosine-5`-triphosphate
APF	antiproliferative factor
BCG	bacillus Calmette-Guérin
BrdU	bromdeoxyuridin
CYP	cyclophosphamide
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFr	epidermal grow factor receptor
HB-EGF	heparin-binding EGF
HER	human epidermal grow factor receptor
IC	interstitial cystitis
LPS	lipopolysaccharide
MAP	mitogen-activated protein
PCNA	proliferating cell nuclear antigen
PKC	protein kinase C
TBS	tris-buffered saline
TGF-α	transforming growth factor-alpha

ABSTRACT

Proliferation of the urothelium, the inner epithelial lining of the bladder is very low under normal conditions but may increase during wound healing and inflammation. In the present study, proliferation in the urinary bladder in response to inflammation was assessed by using two experimental models for cystitis. In order to induce bladder inflammation, cyclophosphamide (CYP; 100 mg/kg intraperitoneally) or E. coli lipopolysaccharide (100 μg/kg intravesically) were administered to female rats. 20 and 56 hours later, rats were administered the synthetic nucleotide bromodeoxyuridine (BrdU, 50 mg/kg) and 4 hours later the rats were killed. In order to detect proliferation in the urothelium, immunohistochemistry was performed on the expression of the proliferation markers Ki-67 and BrdU. Signs of bladder inflammation (bladder wall thickening and hemorrhages) were observed both at 24 and 60 hours after CYP pre-treatment. After 24 hours, proliferation was observed in the urothelial layer of the bladder. After 60 hours, proliferation in the urothelium was decreased but appeared instead in the submucosa. Intravesical administration of LPS failed to induce bladder inflammation. The present study shows that the urinary bladder may attain a dynamic regenerative capacity in response to inflammation. Further studies are needed to assess which cells that are proliferating in the mucosa and submucosa during inflammation.

ABSTRAKT

Proliferace urothelia, vnitřní epiteliální vrstvy močového měchýře, je za normálních podmínek velmi pomalá, ale během hojení ran nebo zánětu se výrazně zvyšuje. V této studii byla hodnocena proliferace buněk močového měchýře jako odpověď na zánět za použití dvou experimentálních modelů pro cystitidu. K vyvolání zánětu močového měchýře byl použit cyklofosfamid (CYP, 100 mg/kg intraperitoneálně) nebo lipopolysacharid E. coli (LPS, 100 μg/kg intravesikálně), které byly podány samicím potkanů. O 20 a 56 hodin později, byl zvířatům podán syntetický nukleotid bromodeoxyuridin (BrdU, 50 mg/kg) a 4 hodiny poté byla zvířata utracena. Pro detekci proliferace urothelia byla provedena imunohistochemie za použití proliferačních markerů Ki-67 a BrdU. Známky zánětu, jako zesílení stěny močového měchýře a hemoragie, byly pozorovány po 24 i 60 hodinách od podání dávky CYP. Po 24 hodinách se zvýšená proliferace objevila v epiteliální vrstvě močového měchýře, po 60 hodinách se proliferace urothelia snížila, ale naopak se zdála být vyšší v submukóze. Intravesikálním podáním LPS se nepodařilo vyvolat zánětlivou reakci močového měchýře. Tato studie ukazuje, že během zánětu má močový měchýř dobrou regenerační schopnost. Pro zjištění které buňky proliferují v mukóze a submukóze během zánětu, jsou potřebné další studie.

INTRODUCTION

Anatomy and histology of the lower urinary tract

To maintain the homeostasis in human organism the kidneys excrete urine that differs markedly from the composition of plasma. The osmolality of urine may be ranging from 50 to 1,200 mosmol/kgH_2O, pH values between 4.5 and 10.0, and levels of ammonia, sodium, potassium, urea, and other toxins are much higher than the levels of these substances in plasma. Urine is secreted from the kidneys and passed through the ureters to the urinary bladder where it is stored (Lavelle et al., 2002, Zeidel, 1996). The urinary bladder is a hollow muscular organ that has more or less spherical shape and is located in the abdomen right behind the pubic bone. The human urinary bladder may contain up to one litre of urine. Both ureters enter the bladder from the sides and go diagonally through the bladder wall. During bladder filling, the orifices of the ureters are squeezed, which prohibits reflux of urine from the bladder to the kidneys. The orifices of the ureters are located near the bladder outlet and a triangle is thus formed between the two ureteral openings and the bladder outlet that is called the trigone (Martinni et al., 2000).

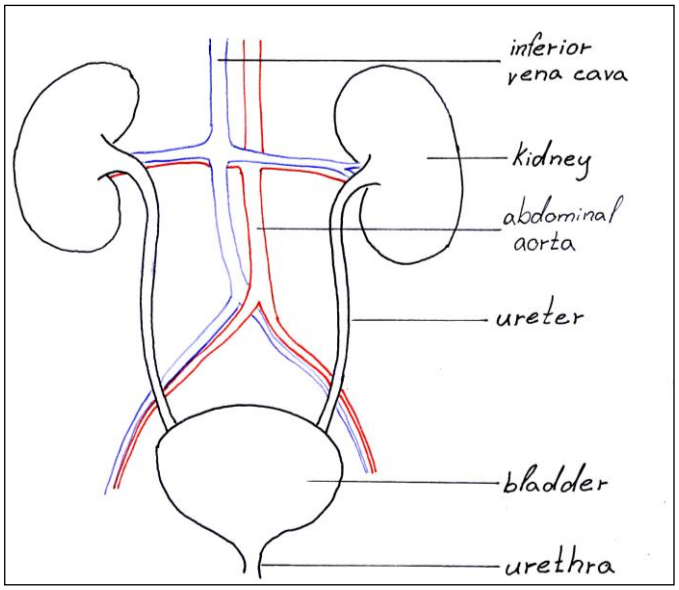


FIG. 1 - Scheme of urinary tract

The bladder wall is composed of four layers: the serous, muscular, submucous, and mucous layer. The serous layer (*tunica serosa*) is derived from the peritoneum. It covers the superior surface and the upper parts of the lateral surfaces and is reflected onto the abdominal and pelvic walls. The muscular layer (*tunica muscularis*) consists of an inner and outer longitudinal smooth muscle layer and a circular layer between. These layers form the detrusor muscle, and when it contracts urine is consequently expelled into the urethra. In the lower part of the bladder, around the internal urethral orifice, the smooth muscle fibres are disposed in a thick circular layer, forming the sphincter vesicae (Martinni et al., 2000). The detrusor muscle of the rat bladder is innervated almost exclusively by cholinergic endings, and adrenergic nerve endings are rare (Watanabe and Yamamoto, 1979).

The submucous layer (*tela submucosa*) consists of areolar tissue and interconnects the muscular and mucous layer. The mucous layer (*tunica mucosa*) is thin, smooth, and of a pink colour. It is continuous with the mucosa of the ureters and renal tubules, and with that of the urethra. The mucosa consists of the urothelium and the basal membrane (Martinni et al., 2000).

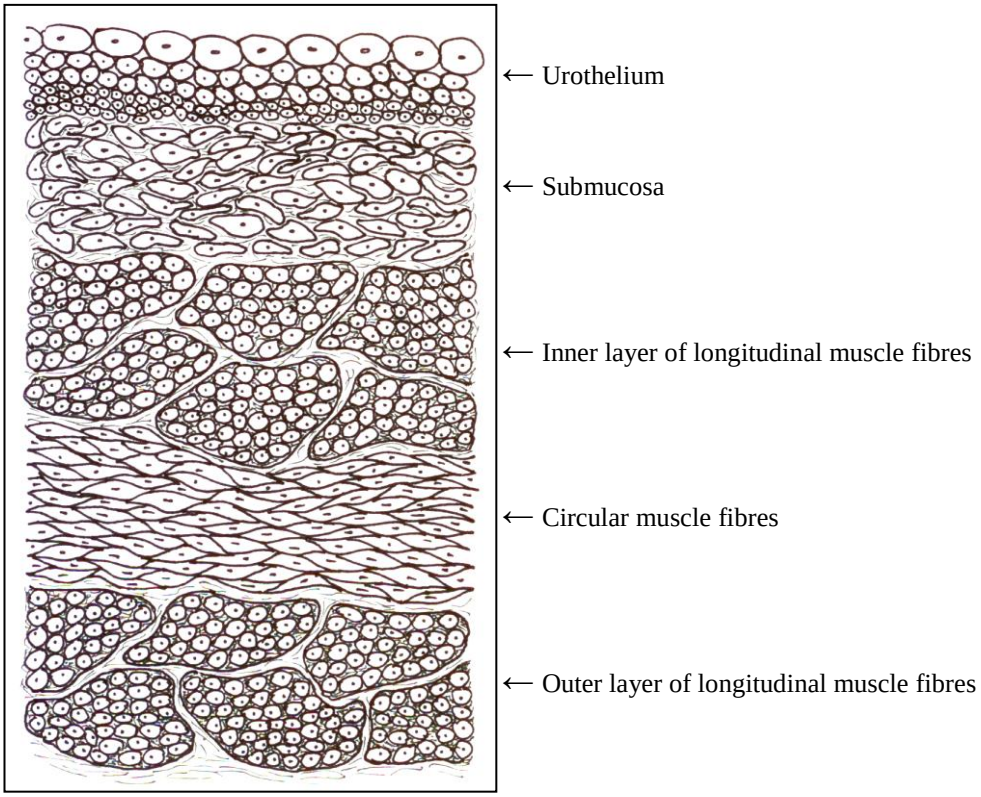


FIG. 2– Vertical section of bladder wall

The urothelium covering the luminal side of the urinary bladder is the epithelial lining of the urinary tract. It is composed of small cells in the basal layer (5–10 μm in diameter), moderately-sized cells in the intermediate layer (20 μm in diameter) and large umbrella cells that form the superficial layer lining of the bladder surface. The size of the umbrella cells depends on the degree of bladder stretch and may vary from 25μm to 250μm in diameter (Apodaca, 2004). Umbrella cells modulate their apical plasma membrane surface area in response to hydrostatic pressure (Wang et al., 2003). Biotinylation experiments demonstrated that the amount of uroplakin III (a molecule found in discoidal vesicles) at the cell surface significantly increased after 5 hours of stretch, consistent with discoidal vesicle fusion. Exocytosis and endocytosis increase during stretching of umbrella cells and the endocytosed membrane is delivered to lysosomes and degraded by a leupeptin-sensitive pathway. Stretch also stimulates ion transport (Truschel et al., 2002).

Barrier function of the urothelium

The mammalian urothelium is one of the most efficient impermeable barriers known to exist in nature. It maintains a high concentration gradient of ions between the urine and plasma. It is impermeable to highly permeable molecules such as water, urea, ammonium and others small nonelectrolytes (Chang et al., 1994). The impermeability of the urothelium depends on three components: the apical membrane, the tight junctions, and a trafficking mechanism that inserts and removes apical membrane in response to filling and emptying of the bladder. (Lavelle et al., 1998). The apical membrane of the umbrella cells is covered by plaques consisting of 16 nm uroplakin particles. These particles are transmembrane proteins, which together with specialized lipids help to create the impermeability of the urothelium. Uroplakins may also act as docking sites for bacterias that cause urinary tract infections. Each uroplakin particle has a central, 6 nm, lipid-filled hole, surrounded by six inverted U-shaped subunits. Uroplakins may therefore severely restrict the movement of membrane lipids, which might contribute to its barrier function (Min et al., 2003, Lavelle et al., 2002, Hu et al., 2002).

When the barrier function of the urothelium is damaged, urine components leak into the underlying layers of the urinary bladder and symptoms of cystitis such as frequency, urgency, and bladder pain may evolve. Disruption of the urothelial barrier may be evoked for example by infection, exposure to chemicals or tumour growth (Lavelle et al., 2002, Lavelle et al., 1998).

Sensory properties of the urothelium

A few years ago it was thought that the urothelium works only as a passive barrier, but during the last years it has been demonstrated that it is involved in antigen presentation and has sensory transduction properties (de Groat, 2004). Urothelial cells may respond to chemical and physical environments and urothelial cells may communicate with neighbouring cells and nerves within the bladder wall (Birder, 2005). Several substances are released from urothelial cells following physical and chemical stimulation, *e.g.*, adenosine-5`-triphosphate (ATP); nitric oxide (NO); substance P; acetylcholine, adenosine, antiproliferative factor (APF), cytokines, prostanoids and various trophic factors. The urothelium is populated by numerous receptors, for example receptors for bradykinin, neurotrophins, purines, norepinephrine, acetylcholine (nicotinic and muscarinic receptors), protease-activated receptors, amiloride-sensitive and mechanosensitive Na^+ channels, and a number of transient receptor potential channels (Birder and de Groat, 2007). The bladder urothelium can serve as a sensor of bladder filling by releasing ATP. Human bladder urothelial cells can release ATP in response to mechanical stretch (Ferguson et al., 1997). An augmented extracellular ATP signalling in bladder urothelial cells could explain the hypersensibility of the bladder in patients with interstitial cystitis (IC). The purinergic signalling pathway in urothelial cells has been suggested as a target in the treatment of hypersensory bladder dysfunction such as IC (Sun & Chai, 2006).

Proliferation of the urothelium

In the normal bladder, the urothelium is one of the slowest growing epithelia. Surgical and traumatic injuries to the bladder initiate a complex series of biological processes that result in wound healing. This process involves cellular proliferation, migration and differentiation, removal of damaged tissue; and production of extracellular matrix, all of which may be controlled by growth factors. During wound healing proliferation of the urothelium may increase rapidly (Cheng et al., 2002). Proliferation increases already eight hours after injury and peaks at 16 hours. Four hours after the injury, a re-epithelization may be observed in wounds where the urothelium is initially completely detached. This process starts adjacent to intact mucosa and is completed after 24 hours. After three days the urothelium is hyperplastic. The basal stem cells are divided to form a hyperplastic epithelium composed of many layers of undifferentiated, predominantly diploid cells. This compensatory proliferation is reversible and is normalized after 5 to 10 days of repair. At day 10, hyperplasia regresses to form three- to four-layered polyploid urothelium with the normal differentiation of intact urothelium. In contrast, pre-neoplastic hyperplasia of the urothelium is irreversible, and the proliferation continues and tumours are consequently formed. In the early stages of growth, pre-neoplastic hyperplasia is histologically very similar to benign hyperplasia (Hicks and Wakefield, 1976, de Boer et al., 1994). Inflammation may also significantly augment the proliferative rate of the urothelium (Limas, 1993).

Normal proliferation is regulated by growth factors and their receptors. Growth factors and their transmembrane receptor kinases play important roles in cell proliferation, survival, adhesion, migration and differentiation (Yarden and Sliwkowski, 2001). These factors may also be involved in any of the several steps in the neoplastic development and progression. The mechanisms by which growth factor receptors participate in malignant transformation include receptor activation by mutation, autocrine and paracrine growth loops, changes in signalling and regulatory pathways and, possibly, receptor trans-activation (Aaronson, 1991, Kolibaba and Druker, 1997). Exogenously added amphiregulin, epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α) and heparin-binding EGF (HB-EGF) stimulate urothelial regeneration (Daher et al., 2003). Protein kinase C (PKC) includes a family of at least 11 serine-threonine kinases. Sustained activation of PKC has been suggested to induce proliferation, differentiation, apoptosis,

migration, or tumorigenesis (Koivunen et al. 2006). PKC-α and -ßI expression and cPKC activity are increased in transitional cell carcinoma (TCC) tumours, particularly in the most proliferative areas of the tumour. In normal epithelium, a significant number of cells were negative for PKC-ßI nuclear localization, whereas in cancerous tissue, particularly in highly proliferative areas, nearly all nuclei were positive. The evidence of Aaltonen's study suggests that PKC-α and -ßI expression in TCC is under control, perhaps of some external or internal stimulus, and suggests that PKC- α and -ßI expression and activity take part in increased growth of TCC **(**Aaltonen et al., 2006).

Bladder cancer

Carcinoma of the urinary bladder is the most common malignancy in the Middle East and parts of Africa where schistosomiasis is a widespread problem. Much evidence supports the association between schistosomiasis and bladder cancer (Mostafa et al., 1999). In Western countries, bladder cancer is the fourth and fifth most common neoplasm among men and women, respectively. About 90% of all bladder cancers arise in the urothelium (el-Marjou et al., 2000). Urothelial tumours are classified into two groups based on histopathology and clinical manifestation, *i.e.*, non-muscle invasive and muscle-invasive tumours. More than 80% of bladder tumours are non-muscle invasive papillary tumors. Patients with such superficial tumours frequently develop recurrences (approximately 70%) often over the course of many years but progression to muscle invasion occurs in only 10–20% of cases. Muscle invasive urothelial tumours have a much less favourable prognosis and often progress rapidly (Knowles, 2001). Cell must acquire a series of traits in order to become malignant and to develop advanced lethal cancers. These traits are: growth factor independence, insensivity to antiproliferative signals, escape from apoptosis, unlimited proliferative capacity, angiogenesis and ability for invasion and metastasis (Bellmunt et al., 2003).

WHO 2004 classification of noninvasive and invasive urothelial neoplasms.

Noninvasive urothelial neoplasms
Hyperplasia (flat and papillary)
Reactive atypia
Atypia of unknown significance
Urothelial dysplasia (low-grade intraurothelial neoplasm)
Urothelial *carcinoma in situ* (high-grade intraurothelial neoplasm)
Urothelial papilloma
Inverted type urothelial papilloma
Papillary urothelial neoplasm of low malignant potential
Noninvasive low-grade papillary urothelial carcinoma
Noninvasive high-grade papillary urothelial carcinoma
Invasive urothelial neoplasms
Invasion of the lamina propria
Invasion of the muscularis propria (detrusor muscle)

(Álvarez Kindelán J. et al, 2007)

Risk factors for the development of bladder cancer

There are several factors identified as risk factors in the development of bladder cancer. Cigarette smoking is the most important risk factor and the risk enhances with the number of cigarettes smoked daily and number of years of smoking (Brennan et al., 2000). Chronic low-dose radiation could also affect bladder urothelium through oxidative stress and impairment of DNA repair and thereby increase the risk of bladder cancer (Brenner et al., 2000). Exposure of aromatic amines or nitrosamines, chronic inflammation, and the cytostatic drug cyclophosphamide (CYP) may also lead to the development of bladder cancer (Murta-Nascimento et al., 2007). Inflammation is a response to injury caused by chemical substances, mechanical stimulus, microorganisms or other agents. Bladder inflammation is often caused by bacteries (*e.g.* Escherichia coli) and women are particularly susceptible to cystitis. This is due to that bacterias often ascend from the mucous layer of the perineal area to the bladder and women have shorter urethra than men (Bednář et al. 1982). During inflammation, tryptase and other serine proteases such as thrombin and trypsin are produced. Tissue responses to these enzymes are modulated by protease-activated receptors, which belong to the G-protein coupled receptors (Saban et al., 2007). Rosin summarized some of the first evidence supporting an association between chronic inflammation in humans and an elevated level of genetic damage in the inflamed

site, thus inflammatory cells may increase genetic damage through enhanced stimulation of cell proliferation (Rosin et al., 1994a). Activated inflammatory cells may induce the development of micronuclei in bladder cells, and this response is associated to chromosome 11, a chromosome commonly altered during bladder carcinogenesis (Rosin et al., 1994b). Bladder carcinoma cells are sensitive to induction of micronuclei by promoter-activated neutrophils (Rosin et al., 1994a).

The role of growth factors in bladder cancer

Many human tumours express high levels of growth factors. Expression of tyrosin-kinase receptors, including many growth factor receptors, has been discovered in urothelial tumours. Systemic administration of growth factor inhibitors keeps down the growth and metastasis of human transitional cells (Bellmunt et al., 2003, el-Marjou et al., 2000). The EGF receptor (EGFr) family consists of four transmembrane tyrosin kinase receptors – the epidermal growth factor receptor EGFr (human epidermal grow factor receptor 1 - HER1), HER2, HER3, and HER4 (Yarden and Sliwkowski, 2001) and at least nine structurally similar peptides that may activate one or more of the four transmembrane receptors of the family. The family is responsible for fetal development and for tissue repair in the adult organism (Nexo, 1994). The receptors are situated at the cell membrane. Each monomer consists of an extracellular ligand-binding domain, a single transmembrane domain and an intracellular domain with kinase activity. Binding of the appropriate growth factor leads to receptor homo- and heterodimerization, activation of the intrinsic kinase domain and phosphorylation of specific tyrosine residues within the cytoplasmic tail. Proteins dock on these phosphorylated residues, leading to the activation of a variety of intracellular signalling pathways. The signal propagated to the nucleus induces altered expression of target genes that promote cell growth, proliferation, differentiation, and migration (Baselga and C.L. Arteaga, 2005, Olayioye et al., 2000). HER2 is up-regulated in the cancerous bladder but does not discriminate between bladder cancer with or without metastases. HER4 is important for differentiation in normal urothelium (Røtterud et al., 2005). The EGFr is an important member of the receptor tyrosine kinase family and is a transmembrane glycoprotein that carries oligosaccharide chains, some of which are similar to blood group determinants (Limas, 1991). The EGFr is able to react with several ligands *i.e.* EGF, HB-EGF, transforming growth factor α and amphiregulin. Intracellular pathways corelated to the EGFr are several and the most important and best understood is the Ras-

MAP (mitogen-activated protein) kinase pathway, which is critical for EGFr-induced cell proliferation. Over-expression of the EGFr dramatically increases growth of the urothelium, leading to hyperplasia (Olayioye et al., 2000). EGFr may cooperate with oncogenes to accelerate tumour growth; EGFr over-expression is, however insufficient for bladder tumours to become invasive. The development of bladder cancer is multifactorial and other events are also necessary for increasing tumorigenesis, which may include inactivation of additional tumour suppressor genes, activation of other oncogenes, down-regulation of cell adhesion molecules, and over-expression of vascular growth factors, cyclooxygenases, and matrix-degrading enzymes (Cheng et al., 2002).

Umbrella cells protect the interaction between EGF and its receptors in normal conditions, but during urothelial tumorigenesis the urothelial permeability is transformed and the EGFr is over-expressed. In Messing's study concentrations of EGF were found to be significantly reduced in group of patients with bladder cancer. Although no given concentration of urinary EGF is diagnostic for the existence or absence of transitional carcinoma cells, the significantly reduced concentration in patients with bladder tumours is consistent with the concept that urinary EGF is abnormally bound to malignant and premalignant urothelium (Messing et al., 1994). Excessive production (*e.g.* in bladder tumours) or application of EGF, TGF-α or amphiregulin may lead to either hyperplasia or a faster repair of damaged urothelium *in vivo* (Brown et al., 1989). These results suggest that modified members of the EGF family are potential targets for future therapies for bladder wound healing and malignancy (Bindels et al., 2002). EGFr expression is significantly correlated with disease-specific mortality among patients with bladder cancer. However, cytoplasmic HB-EGF expression indicated an improved prognosis even in the case of strong EGFr expression. In contrast, nuclear HB-EGF and strong EGFr expression was associated with an extremely poor prognosis (Kramer et al, 2007).

HB-EGF is a member of the EGF family and is a 76–86 amino acid glycosylated protein. HB-EGF is an activating ligand for HER1 and a potent mitogen for smooth muscle cells and fibroblasts (Yoshizumi et al., 1992, Dluz et al., 1993). HB-EGF stimulates the growth of a variety of cells in an autocrine or paracrine manner and is known to be up-regulated in the wound-healing process of certain cell types. HB-EGF exerts oncogenic potentials *in vitro* and *in vivo,* and HB-EGF also increases tumour angiogenesis. Thus HB-EGF may constitute an important target in cancer treatment (Ongusaha et al, 2004).

Therapy of bladder cancer

Immunotherapy may be used in bladder carcinoma treatment. Intravesical bacillus Calmette-Guérin (BCG) is an immunotherapeutic agent that when given intravesically is very effective in the treatment of superficial TCC. Compared with controls, BCG has a 43% advantage in preventing tumour recurrence, a significantly better rate than the 16% to 21% advantage of intravesical chemotherapy. In addition, BCG is particularly effective in the treatment of carcinoma in situ, eradicating it in more than 80% of cases (Amling, 2001). Intravesical BCG immunotherapy remains the most effective treatment and prophylaxis for TCC and has positive outcomes on tumour recurrence rate, disease progression, and prolongation of survival. Prostatic urethral mucosal involvement with bladder cancer can be effectively treated with BCG intravesical immunotherapy; it has been demonstrated a reduction in tumour recurrence rates (Nseyo and Lamm, 1997). The exact antitumour mechanism of BCG is not clear but it is known that a complex cascade of immunomodulating processes is involved. One of the main aims of treating patients with intravesical BCG is to reduce the recurrence and progression rate. The response depends on the stage and grade of tumour. BCG has been shown to be superior to many of the intravesical chemotherapy agents used (Razack 2007).

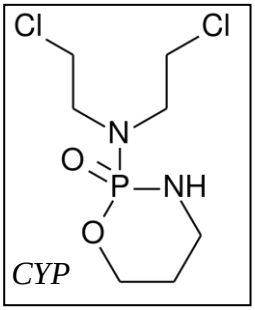
The treatment of muscle-invasive bladder carcinoma includes eradication of local disease, elimination of potential micrometastases and maintenance of quality of life without compromising survival. The standard treatment of muscle-invasive bladder carcinoma is radical cystectomy (Ruggeri et al., 2006). While radical cystectomy cures many patients, almost 50% of patients develop metastatic disease (Scher et al., 1989). Adjuvant chemotherapy has been proposed to reduce the probability of relapse and to improve survival (Dimopoulos and Moulopoulos, 1998). The use of neoadjuvant cytostatic drug such as methotrexate, vinblastine, doxorubicin, and cisplatin followed by radical cystectomy is associated with improved survival among patients with locally advanced bladder cancer (Grossman et al., 2003).

Palliative radiation therapy has been well studied in the setting of bone metastases and treatment of hematuria in locally-advanced bladder cancer. Radiation therapy is used for pain control in patients that have recurrent and locally advanced bladder cancer. Radiation therapy should be offered to patients with medically unmanageable pain,

because pain relief is an important component in the optimization of quality of life (Yi et al., 2007). Radiotherapy and chemotherapy are also suitable after non-radical operation (Visockiene et al., 2004).

The use of monoclonal antibodies or specific inhibitors targeting EGFr function may open up new possibilities for effective treatment. It has been demonstrated that therapy with EGFr inhibitors increases the activity of conventional cytoreductive chemotherapeutics agents, in part by inhibiting tumour cell proliferation, angiogenesis, and inducing apoptosis (Limas, 1991, Bellmunt et al., 2003, el-Marjou et al., 2000). The two most commonly used monoclonal antibodies are cetuximab (monoclonal antibody to the EGFr) and trastuzumab (monoclonal antibody to HER2 receptor), presently these drugs are used in the second and the third phase of clinical trials. Cetuximab induces dimerisation, internalisation and downregulation of the EGFr and blocks activation of the tyrosine kinase domain when the ligand is bound. This agent has activity either alone or in combination with chemotherapy (Goldstein et al., 1995, Reid et al., 2006). The mechanisms of the action of trastuzumab (herceptin) involve disruption of DNA repair and induction of antibody-dependent cellular cytotoxicity (Dank, 2001).

In the present study, animals will be pre-treated with cyclophosphamide (CYP) intraperitoneally and by Escherichia coli lipopolysaccharide (LPS) intravesically in order to induce inflammation of the bladder. CYP is an alkylating agent used in chemotherapy of neoplastic diseases. CYP is carcinogenic and is associated with increased risk of bladder cancer (Murta-Nascimento et al., 2007). CYP is metabolized to cytotoxic substances such as acrolein by the hepatic cytochrome P450 system in the liver and possibly also in the kidney. Metabolites of the drug are excreted in the urine and induce immunoreaction manifested by necrosis and apoptosis of the urothelial layer. Urothelial proliferation occurs already during urothelial necrosis after a single CYP injection, and after 24 hours proliferation is stronger than necrosis (Jezernik et al., 2003, Hardman & Limbird, 2001).



CYP

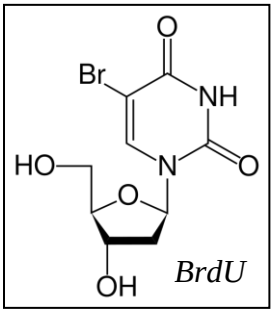
LPS is a major component of the outer membrane of gram-negative bacteria, for example Escherichia Coli. LPS is an endotoxin, and induces a strong immunological response in animals. Intravesically-administred LPS induces an inflammatory response of the bladder and rapidly increases phosphorylation of the Akt/protein kinase B, a key enzyme regulating proliferation, apoptosis, and inflammation (Kang et al., 2004). Oxidative stress by reactive oxygen intermediates and a proliferative response of the carcinogen-exposed urothelium to the inflammatory stimulation appeared to play a significant role in tumour enhancement by LPS, but treatment with LPS alone does not induce tumours (Kawai et al., 1993).

Aims of study

The present study aims at investigating proliferation of the urinary bladder in experimentally-induced cystitis. Cell proliferation will be assessed by immunohistochemistry. Immunohistochemistry is a method of localizing proteins in cells of a tissue section working on the principle of antibodies binding specifically to antigens in biological tissues. Immunohistochemical staining is widely used in the diagnosis of cancer. Visualizing an antibody-antigen interaction can be accomplished in a number of ways. The antibody can be tagged to a fluorophore; this method is used in confocal laser scanning microscopy, which is highly sensitive and can also be used to visualize interactions

between multiple proteins. But most commonly an antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction.

In the present study, expression of the proliferation markers Ki-67 and bromodeoxyuridine (BrdU) will be studied by immunohistochemistry. BrdU is a thymidine analogue that is able to incorporate into replicating DNA of S-phase cells. Antibodies specific for BrdU can then be used to detect the incorporated chemical, thus indicating cells that are actively replicating their DNA. Binding of the antibody requires denaturation of the DNA, usually by exposing the cells to acid or heat. BrdU is used to determine relative proliferation rates, the length of cell cycle, and the percentage of cells in the cell cycle (Tang et al., 2007). Ki-67 is a nuclear antigen expressed during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). Ki-67 index is the percentage of Ki-67 positive tumour cells of the total number of tumour cells. In previous studies it has been demonstrated that the frequency of Ki-67-positive tumour cells increased with the tumour grade and stage of bladder carcinoma (Bush et al., 1991, Fontana et al., 1992).



BrdU

METHODS

Animals and pre-treatments

The ethical committee of Gothenburg University approved the study design, in which six female Sprague-Dawley rats (200-250g) were pre-treated with CYP (100mg/kg) intraperitoneally and by LPS (100 μg/kg) intravesically in order to induce inflammation. Some animals were pretreated with saline (0.9% solution of NaCl) intraperitoneally serving as controls. 20 and 56 hours later, rats were administered the synthetic nucleotide bromodeoxyuridine (BrdU 50 mg/kg) and 4 hours later the animals were anaesthetized with pentobarbitone (50 mg/kg) intraperitoneally and the urinary bladder and jejunum were consequently excised. The animals were consequently killed with an overdose of pentobarbitone. The organs were stored in formaldehyde for 48 hours.

Preparations of bladder sections

Preparations were left in 70% ethanol overnight. The preparations were then dehydrated for 8 hours in ethanol (95% - 99.9%) and xylene. The sections were incubated overnight in 60˚C paraffin and then embedded in paraffin. The paraffin embeddings were sliced by a microtome (4 μm).

Immunohistochemistry

One part of this project was to develop the protocol for immunohistochemistry, the final version of the protocol was the following:

Sections were first incubated at 60˚C for one hour. The sections were then deparaffinated with xylene and decreasing concentrations of ethanol (99.5-70%, 10 min in each xylene/ethanol bath). Sections were washed in tris-buffered saline (TBS) and were then boiled in citrate buffer (pH 7.5) four times for 6 min. Endogenous peroxidase was inactivated incubating the sections in 0.3% H_2O_2 incubation for 30 min. Then sections were consequently washed in TBS and incubated for 30 minutes with 2M HCl in 37˚C to

denature histones and DNA. The pH was consequently neutralized by 0.1 M borate buffer (pH 8.5) and sections were washed in TBS again, followed by 5% TBS-plus (TBS with 5% of horse serum to block unspecific binding sites and with Triton-X detergent that makes it possible for the antibodies to penetrate the membranes of the cells). Sections were incubated with a primary antibody (Monoclonal anti BrdU, antibody produced in mouse, Sigma-Aldrich, Sweden) 1:400 in 1% TBS-Plus or Monoclonal anti-proliferating cell protein Ki-67, antibody produced in mouse (Sigma-Aldrich, St Louis, MO, 1:150 in 1% TBS-Plus) overnight at 4˚C.

The following day the sections were washed in TBS and then in 1% TBS-plus. Consequently the sections were incubated with the secondary antibody (Rabbit ABC staining system: sc-2018, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:150 in 1% TBS-Plus for two hours.

After incubation with the secondary antibody sections were washed in TBS and incubated with ABC-Elite solution (rabbit ABC staining system: sc-2018, Santa Cruz Biotechnology; Santa Cruz, US) for one hour and then washed in TBS again. After 5 min treatment with DAB solution the sections were washed in tap water, colored with hematoxylin and washed in 37˚C water and then in TBS. Thereafter the dehydratation of the sections was performed by incubating the sections in increasing concentrations of ethanol (70-99.5%) and xylene (10 min in each bath). Finally glasses were stuck upon sections. As a negative control, the primary antibody (Ki-67 or BrdU) was omitted and the experimental procedure was then followed as above.

MATERIALS

Materials and substances used in the immunohistochemistry protocol were as follows:

Albumin from bovine serum (Sigma-Aldrich)

Citric acid (Sigma – Aldrich), ethanol (Kemetyl, Haninge, Sweden)

Hydrochoric acid (Sigma-Aldrich)

Hydrogen peroxid (Sigma-Aldrich)

Mayer's hematoxyline (Histolab)

Monoclonal anti BrdU, antibody produced in mouse (Sigma-Aldrich)

Monoclonal anti proliferating cell protein Ki-67, antibody produced in mouse (Sigma-Aldrich)

Pertex (Histolab, Gothenburg, Sweden)

Rabbit ABC staining system: sc-2018 (Santa Cruz Biotechnology; Santa Cruz, US)

Sigma fast 3, 3`- diaminobenzidine tablet sets (Sigma-Aldrich)

Sodium chloride (Sigma – Aldrich)

Sodium hydroxid (Sigma-Aldrich)

Sodium tetraborat 99% (Sigma-Aldrich)

Trition X-100 (Sigma-Aldrich)

Trizmabase (Sigma – Aldrich)

Vectastain – ABC kit (vector laboratories)

Xylene (Histolab)

RESULTS

Development of immunohistochemistry protocol

In order to block unspecific protein binding, incubation of tissue sections were first incubated with goat serum. Since blocking failed, goat serum was changed to horse serum. Different concentrations of horse serum were also used on tissue sections in order to optimize the protocol. Hematoxylin was first used as background staining. In order to better observe specific staining for ki67 and BrdU this step was, however, omitted in the final protocol. Unspecific staining was presently observed for ki67 and BrdU when incubating the sections with DAB for 7 min. Therefore, incubation with DAB was decreased to 3 min.

Immunohistochemistry

Staining for Ki67 (1:150) revealed that after 24 hours of CYP-induced cystitis, proliferation occurred in the urothelial layer of the bladder (Fig. 1-3). After 60 hours of CYP-induced cystitis, proliferation appeared also in the submucosa, while proliferation was diminished in the urothelium (Fig 4-6). In contrast, no significant proliferation was detected in the normal bladder. Immunostaining for BrdU (1:400) revealed an abundant proliferation in ileal sections (see fig. 7), but staining was in contrast unsuccessful in bladder sections of CYP-inflamed bladders. The administration of LPS intravesically to rats was unsuccessful since no signs of inflammation were discovered when bladders were consequently examined macro- as well as microscopically.

Figure 3 - 5: Immunohistochemical staining for Ki-67 (diluted 1:150) of sections of urinary bladder from a rat pre-treated with CYP for 24 hours

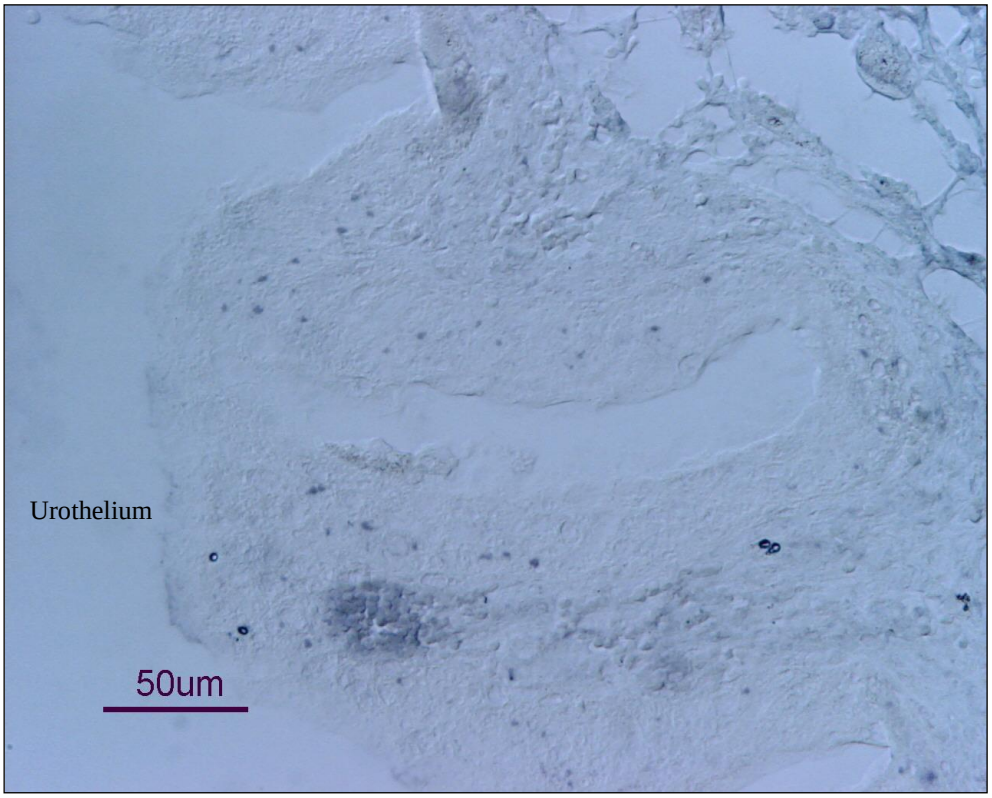


FIG. 3

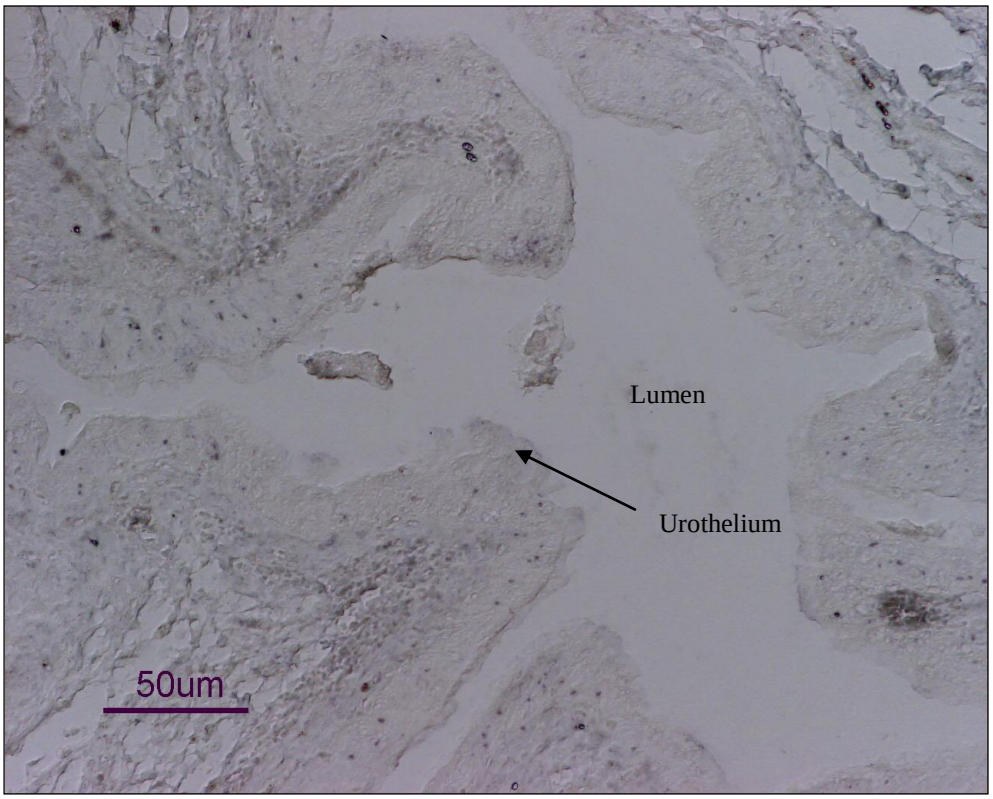


FIG. 4

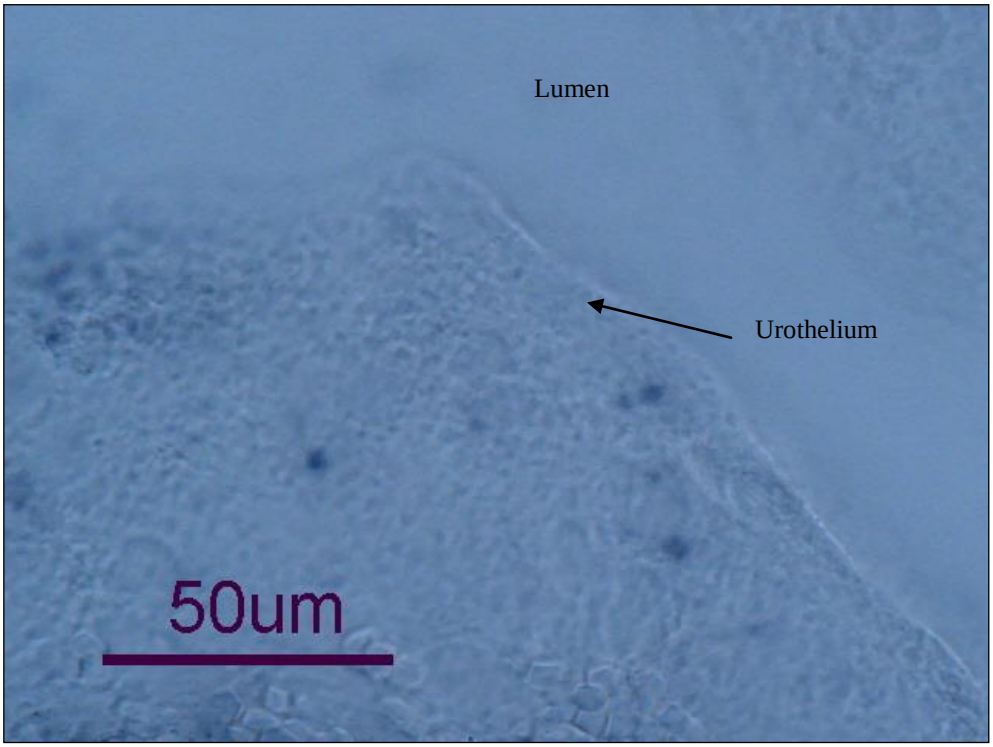


FIG. 5

Figure 6 - 8: Immunohistochemical staining for Ki-67 (diluted 1:150) of sections of urinary bladder from a rat pre-treated with CYP for 60 hours (a-c)

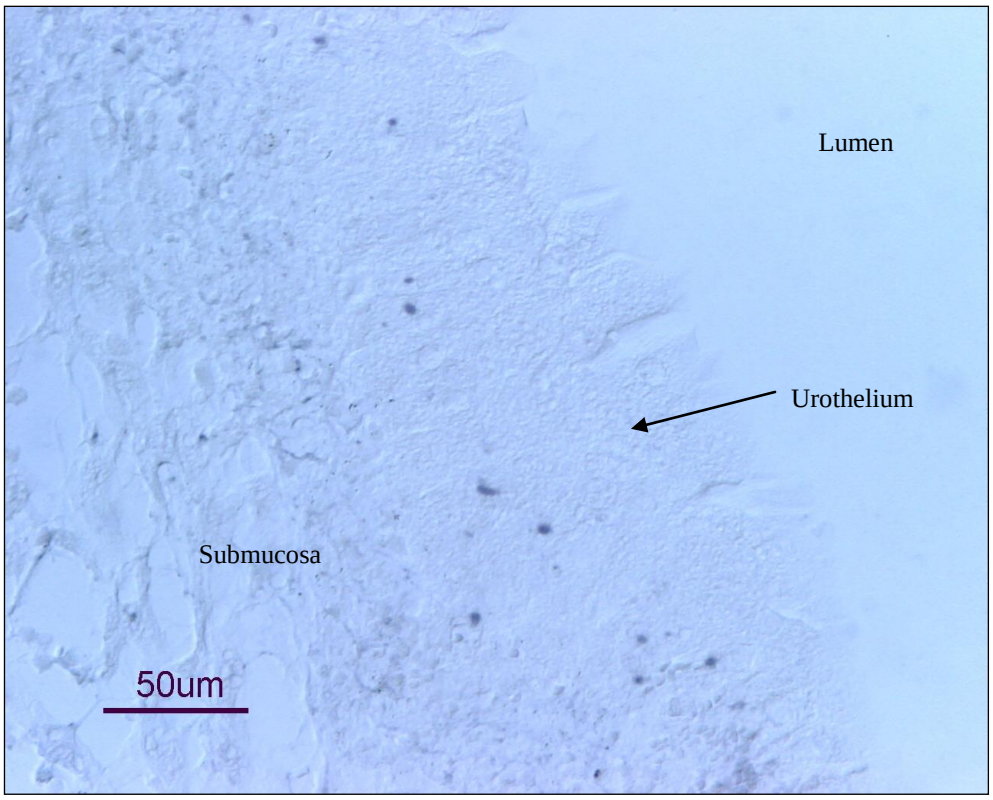


FIG. 6

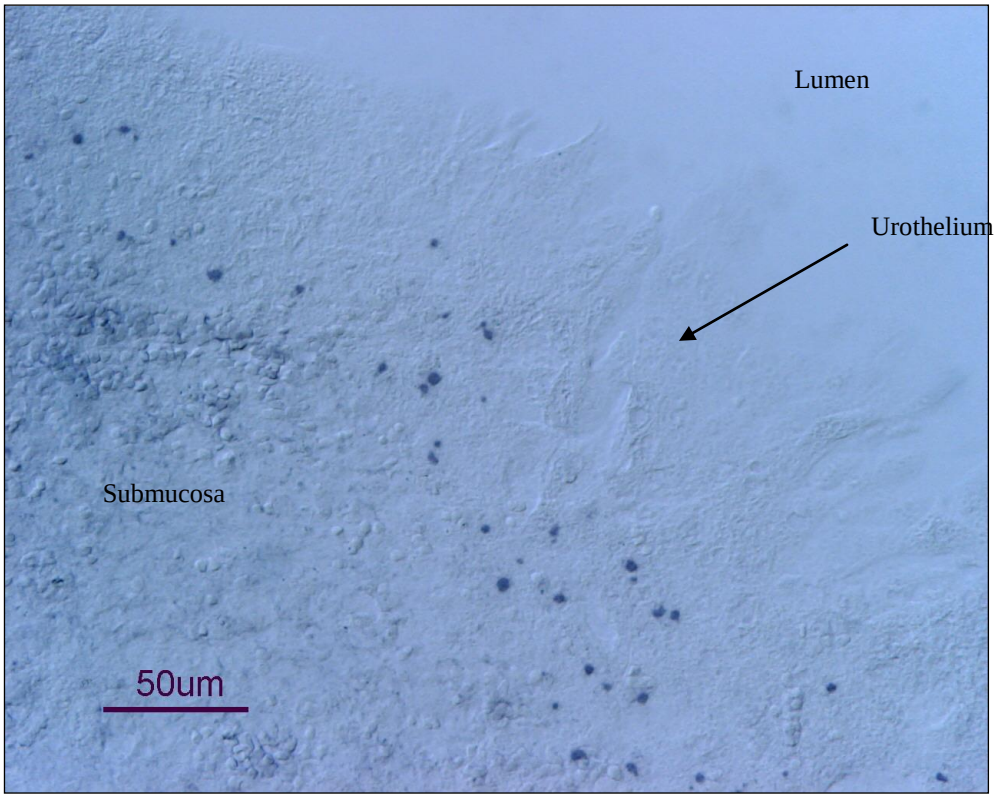


FIG. 7

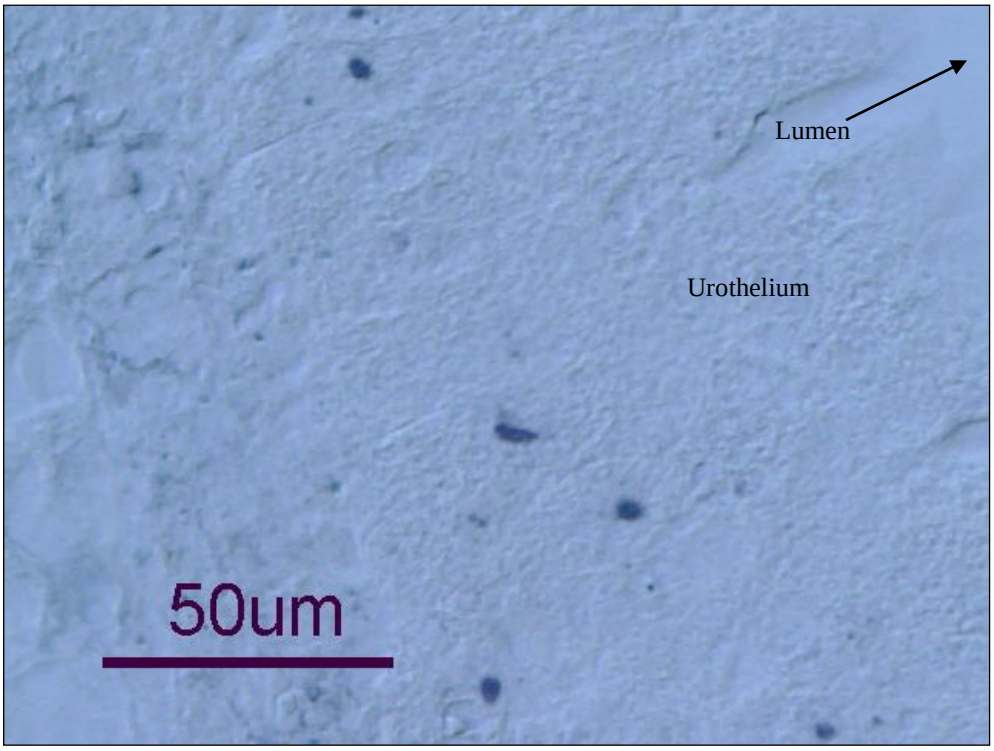


FIG. 8

Figure 9. Immunohistochemical staining for BrdU (diluted 1:400) of a section of ileum from a rat pre-treated with saline.

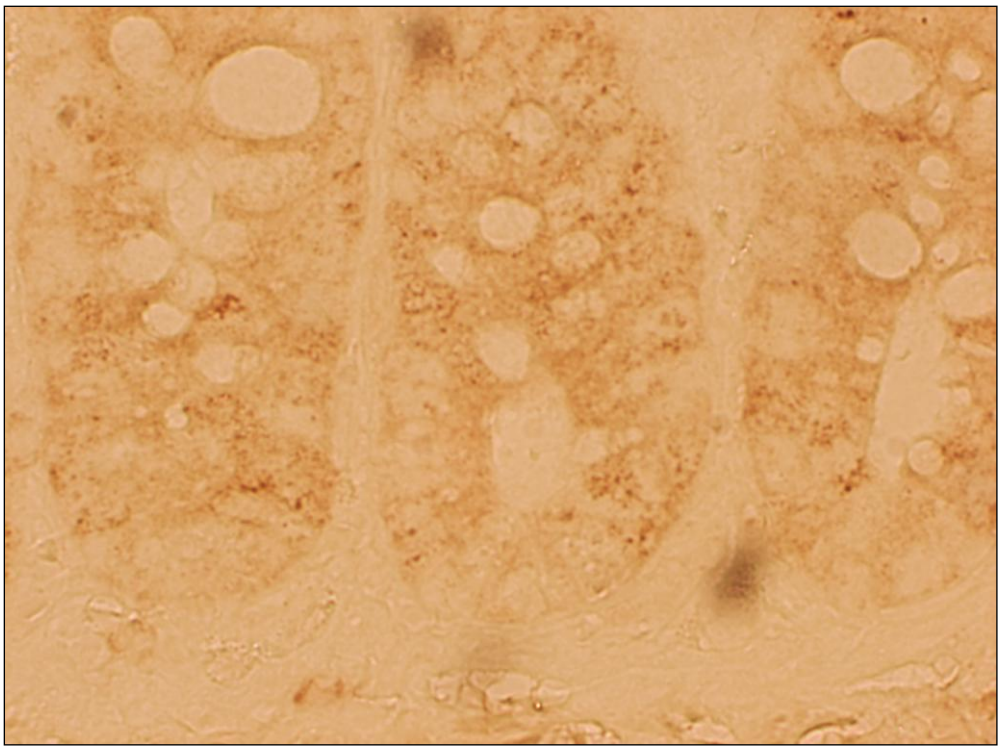


FIG. 9

DISCUSSION

In the present study, cell proliferation of the urothelium during experimentally-induced cystitis was assessed. Proliferating urothelial cells are rare in the normal urinary bladder (Jezernik et al, 2003, Romih et al, 2001). In the state of CYP-induced cystitis, both cell apoptosis and proliferation occur in the urothelium (Jezernik et al, 2003). Proliferation in the urothelium after CYP treatment, assessed by staining cells for proliferating cell nuclear antigen (PCNA), occurs already after 6 hours and increases during the first 24 hours, while apoptosis appears 12 hours and is reduced 18 hours after CYP administration (Jezernik et al, 2003). Proliferation first takes place in the basal layer of the urothelium. (Jezernik et al. 2003), Romih et al., 2001) 3 to 5 days after administration of CYP, proliferation occurs also in intermediate and superficial layers of the hyperplastic urothelium. 10 days after administration of CYP proliferation is normalized in the urothelium (Romih et al., 2001). The present results showed that proliferating cells (Ki-67 positive cells) occur in the urothelium 24 h after CYP pre-treatment together with hyperplasia of the urothelium. In contrast to the studies by Romih et al, Ki-67 positive cells were detected in all layers of the urothelium. 60 h after CYP administration, proliferation in the urothelium was reduced, but proliferating cells appeared instead in the submucosa. The type of cell(s) that is proliferating in the submucosa is presently unknown. However, since bladder inflammation peaks at this interval after CYP administration (Souza-Fiho et al., 1997, Giglio et al. 2005), proliferating inflammatory cells may be a candidate.

While Ki-67 staining was successful on bladder sections in the present study, BrdU staining was instead unsuccessful. The situation was the reversed on ileal sections, where only BrdU staining succeeded. Previous studies have, however, detected proliferating cells in the bladder by using BrdU antibodies. (*i.e.* Limas C., 1993, Ohnishi et al., 2007) The discrepancies in the results may depend on differences in antibodies and concentrations needed for different tissues. The immunohistochemistry protocol for BrdU might also need some changes, such as prolongation of the incubation time of the BrdU antibody. Also insufficient denaturation of the DNA strands in the bladder sections might be a cause of failure; thus, a higher concentration of HCl might be needed.

Previous studies have demonstrated that LPS may induce bladder inflammation when administered intravesically (Kang et al., 2004). Saban provides evidence that LPS is capable of inducing and amplifying bladder inflammation (Saban et al., 2002). In the present study, intravesical administration of LPS failed to induce bladder inflammation. This might depend on a too low concentration of LPS or a too short incubation time of LPS.

In conclusion, the present results demonstrate that an enhanced proliferation of the urothelium occurs during bladder inflammation. Further studies are necessary to assess which cells that are proliferating in the mucosa and submucosa during inflammation.

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