Intraepithelial Expansion of Bladder Cancer

Intra-epitheliale expansie van blaaskanker

Proefschrift

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Abbreviations

AS	antisense oligonucleotide
BrdU	bromodeoxyuridine
CIS	carcinoma *in situ*
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ErbB	erythroblastosis virus gene
FCS	foetal calf serum
HER	human EGF receptor
HRGα	heregulin-α
IEE	intraepithelial expansion
IEN	intraepithelial neoplasia
mRNA	messenger ribonucleic acid
MUT	mutant oligonucleotide
PCR	polymerase chain reaction
PBS	phosphate buffered saline
S	sense oligonucleotide
SCID	severe combined immunodeficiency syndrome
SF	serum free medium
SSCP	single-strand conformation polymorphism
TCC	transitional cell carcinoma
TGFα	transforming growth factor-α
TUR	transurethral resection

Chapter 1

GENERAL INTRODUCTION

The urinary bladder

The urinary bladder is a part of the urinary system. Together with the pyelum, ureters and urethra, the urinary bladder is responsible for the excretion of urine. The urinary bladder functions herein as a temporary reservoir for the urine. Whenever it is necessary the urine in the urinary bladder can be voided. The urinary bladder is characterized by its distensibility. The special anatomical structure of the urinary bladder wall is adapted to fulfill this. The urinary bladder wall consists of three main layers: a smooth muscle layer, a stromal compartment, and a mucosal layer. The smooth muscle layer consists of an external and internal layer of longitudinal fibers, and a middle layer of circular muscle fibers. These smooth muscle layers are necessary for emptying of the bladder. The stromal compartment of the urinary bladder wall consists of loosely packed connective tissue, including bloodvessels and nerve fibers. The mucosal layer (i.e. urothelium or transitional epithelium) is the internal lining of the bladder. In humans, the urothelium consists of five to seven polarized cell layers. In the mouse, it consists of three layers. At the basement membrane side of the urothelium the undifferentiated basal cells are located. These basal cells contain the stem cells of the urothelium. Under normal conditions the cell turnover of the urothelium is very low, but after injury to the bladder wall basal cells start to divide rapidly. At the luminal side of the urothelium, large superficial cells (umbrella cells) cover the urothelial cell layers. These umbrella cells form a barrier between the urine and the body fluids and they are important for the plasticity of the bladder. Between the superficial cell layer and the basal cell layer, the intermediate cell layers are located.

Bladder Cancer

Etiology & Epidemiology

Bladder cancer is the fifth most common malignancy in males in the western society [Parkin *et al.*, 1999]. In 1990, an incidence of 24.2 (male) and 6.6 (female) per 100,000 people was reported in Western Europe [Parkin *et al.*, 1999]. In the Netherlands, an incidence of 23.4 (male) and 4.3 (female) per 100,000 people was reported for 1995 (http://www.ikc.nl/vvik/). The risk for the development of bladder cancer increases with age, with a peak incidence around the seventh decade of life. Epidemiological studies have revealed some important risk factors, like the use of tobacco, exposure to aromatic amines,

chronic urinary tract infection, and infection with *Schistosoma hematobium* [Johansson & Cohen, 1997]. It is estimated that cigarette smoking accounts for 25-60% of bladder cancer cases in industrialized developed countries, and is therefore the most important known risk factor for bladder cancer. [Johansson & Cohen, 1997]. In developing countries, infection with *Schistosoma hematobium* is responsible for ± 75% of the detected cases of squamous cell carcinoma in the bladder.

Pathology

In the western society, more than 95% of the bladder tumors are transitional cell carcinomas (TCC). Other urinary bladder tumors are squamous cell carcinoma, mixed carcinoma, adenocarcinoma, and nonepithelial bladder tumors. TCC (the topic of this thesis) can be further categorized into flat lesions (e.g. carcinoma *in situ*), papillary neoplasms, and invasive neoplasms [Epstein *et al.*, 1998] Histologically, papillary TCC are graded according to the WHO grading system: Grade 1 are papillary structures lined by neoplastic transitional epithelial cells that show minimal nuclear abnormalities and mitoses; in Grade 2 the histological and cytonuclear features are between those of grade 1 and 3; Grade 3 is characterized by significant nuclear abnormalities and high number of mitoses. Non-papillary bladder carcinomas are invasive and usually grade 3. Currently, histological grade and clinico-pathological staging are the best predictors of clinical prognosis [Bane *et al.*, 1996]. Nowadays, the TNM-classification (Fig. 1) is the best known and worldwide most frequently used system for staging of bladder cancer. In the TNM classification the extent of the primary tumor (T), the involvement of regional lymph nodes (N) and the presence or absence of distant metastases are determined.

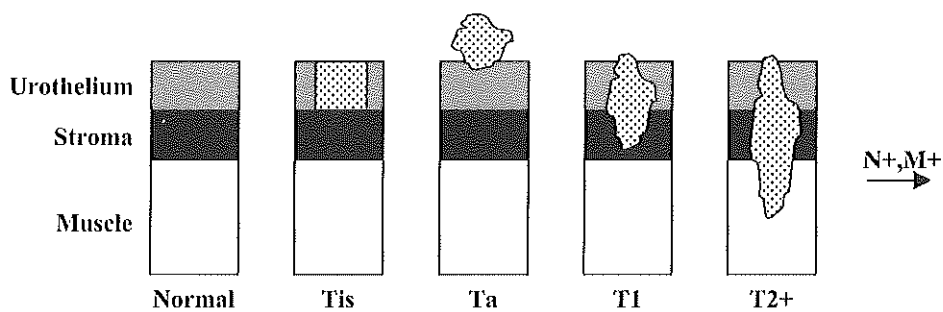

Figure 1: Schematic representation of the TNM system for the classification of bladder cancer.

From a clinical point of view TCC can be separated into superficial tumors and muscle invasive tumors because the natural history and treatment of these two forms are markedly different [Lapham *et al.*, 1997; Raghavan *et al.*, 1990]. More than 70% of patients with TCC are initially diagnosed with superficial tumors (Ta, T1 and Tis). Superficial TCC are treated by transurethral resection (TUR), followed by adjuvant chemo-, or immunotherapy [Soloway, 1992]. Progression to invasive disease is uncommon for patients with superficial TCC, and long-term survival has been achieved in more than 80% of the cases [Raghavan, 1990; Bane, 1996]. On the other hand, patients with muscle invasive TCC (T2+) have a very poor prognosis. The standard primary treatment for locally advanced TCC is radical cystectomy, sometimes in combination with adjuvant chemo-, or immunotherapy [Badalament and Schervish, 1996; Lapham *et al.*, 1997]. Despite these radical therapies, the reported five-year survival rate for invasive bladder carcinoma is less than 50%.

Recurrence of superficial bladder cancer

The major problem in the management of superficial TCC is the high frequency of tumor recurrences. Over 70% of patients with superficial TCC will have one or more recurrences after initial treatment, and one-third of those patients has progression to invasive disease and eventually succumbs to their disease [Heney *et al.*, 1983; Kurth *et al.*, 1989].
Several potential mechanisms may account for the high recurrence rate of bladder cancer:

I) Field cancerization.

An important feature of TCC is its multifocal nature. Biopsies of normal appearing areas in bladders harboring a tumor revealed a high incidence of hyperplasia, atypia, CIS, or cancer [Schade and Swinney, 1973; Heney *et al.*, 1978; Wolf and Højgaard, 1980]. Histological mapping of cystectomy specimens of patients with TCC demonstrated that abnormalities of the surrounding urothelium were common [Farrow, 1976; Koss, 1977a]. These abnormalities ranged from hyperplasia and dysplasia to CIS and carcinoma. Furthermore, Wolf and Højgaard [1983] reported that the presence of urothelial dysplasia concomittant with a bladder tumor is an important determinant for future recurrences. These studies suggested that some of the observed recurrences are actually new primary bladder tumors, derived from areas of atypia or CIS that had progressed. The occurrence of these multiple, abnormal areas in the bladder could be explained by the "field cancerization" hypothesis [Slaughter *et al.*, 1953]. According to this theory, an entire field of tissue (bladder mucosa) is exposed for a long time to carcinogens. The carcinogenic exposure affects multiple cells in the field, which leads to

the occurrence of many genetically unstable cells. Ultimately, proliferation and further progression of such unstable cells can result in aberrant growth and the formation of multiple independent tumors. On the basis of this "field cancerization" hypothesis, some authors suggested that each recurrent bladder tumor had arisen *de novo* from precancerous flat urothelium [Richie *et al.*, 1989].

II) Regrowth of an incompletely resected primary bladder tumor.

Standard treatment of superficial TCC is a local resection of the tumor. If the resection is not complete, this could lead to the outgrowth of residual tumor cells ultimately resulting in a recurrent TCC. Although this seems a very obvious explanation for the appearance of recurrences, it is in contradiction with the observation that most new bladder tumors appear at a site different from the primary tumor [Boyd and Burnand, 1974]. In addition, careful microscopic examination of the scar area after resection of the primary tumor did not reveal residual cancer cells [Badalament, 1996].

III) Implantation of intraluminally dispersed cancer cells (Fig 2A).

Several clinical and experimental studies indicate that recurrences could be the consequence of shedding and subsequent reattachment of tumor cells to intact or traumatized areas of the urothelium.

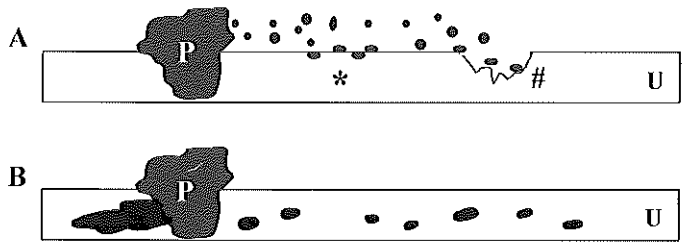

Figure 2: Mechanisms that could be involved in the development of bladder tumor recurrences. A) Intraluminal shedding and subsequent attachment of tumor cells on either intact or traumatized# urothelium B) Lateral intraepithelial spreading of single tumor cells or sheets of bladder tumor cells. U=urothelium, P=primary tumor.*

Hinman (1958) was one of the first to suggest that intraluminally shedded bladder tumor cells could attach to areas in the bladder, remote from the location of the primary tumor. In several *in vivo* models it was shown that bladder tumor cells could attach to traumatized areas in the bladder [Soloway and Masters, 1980; See *et al.*, 1989; See *et al.*, 1990; Hyacinthe *et al.*, 1995]. Although less frequently, *in vivo* attachment of bladder tumor cells to intact areas of the bladder was also reported [Soloway, 1980]. More recently, it was shown that bladder tumor cells are indeed able to attach to and colonize intact urothelium *in vitro* [Rebel *et al.*,

1994a]. The latter study showed that expression of the adhesion molecule E-cadherin by the bladder tumor cells was a requisite for the observed adherence to intact urothelium.

IV) Intraepithelial expansion of cancer cells (Fig 2B).

An alternative mechanism could be lateral expansion of bladder tumor cells into the surrounding normal urothelium [Harris and Neal, 1992; Garcia *et al.*, 1999]. This could either be accomplished by single tumor cells or sheets of tumor cells migrating away from the primary bladder tumor (Fig 2B).

Mechanisms III and IV are supported by recent molecular genetic studies of bladder tumors. These studies revealed that at least in some individuals tumor recurrences are of the same clonal origin. Demonstration of monoclonality in multiple synchronous or metachronous bladder tumors was based on methods such as X-chromosome inactivation, mutation analysis of specific genes (e.g. P53, Rb), and microsatellite analysis [Sidransky *et al.*, 1992; Habuchi *et al.*, 1993; Miyao *et al.*, 1993; Chern *et al.*, 1996; Takahashi *et al.*, 1998]. Monoclonality of these tumours suggested that recurrences could arise by reimplantation or intraepithelial expansion of (residual) tumor cells and subsequent proliferation of bladder tumor cells with selective growth advantages [Foresman and Messing, 1997].

Carcinoma *in situ* of the urinary bladder

Carcinoma *in situ* (CIS) of the bladder can be regarded as an intraepithelial neoplasia (IEN). CIS is a full-thickness malignant (high-grade) change that is confined to the flat (nonpapillary) urothelium (Fig. 3). Morphologically, CIS is characterized by urothelium of variable thickness, which exhibits cellular atypia of the entire mucosa, from the basal layer to the surface [Murphy *et al.*, 1994]. By definition, there is no invasion of the underlying basement membrane. In approximately 10% of CIS a "pagetoid variant" is noted [Orozco *et al.*, 1993]. In this variant individual or small groups of transformed cells are found in the surrounding, normal urothelium of the CIS. CIS is often multifocal, and is usually observed in association with either synchronous or metachronous papillary or invasive TCC. Only in 10% of the patients CIS is diagnosed as the sole lesion in the bladder [Hudson and Herr, 1995]. Although CIS has a cytological resemblance to high-grade carcinoma, expansion occurs only by undermining the adjacent normal urothelium. Even under favorable conditions (e.g. large mucosal defects due to diagnostic biopsies or therapeutic fulguration) the cells of CIS usually do not invade stromal tissue [Murphy, 1994; Orozco *et al.* 1994]. However, at longer time

intervals progression to invasive disease is also frequently reported [Lamm, 1992; Hudson and Herr, 1995; Cheng *et al.*, 1999].

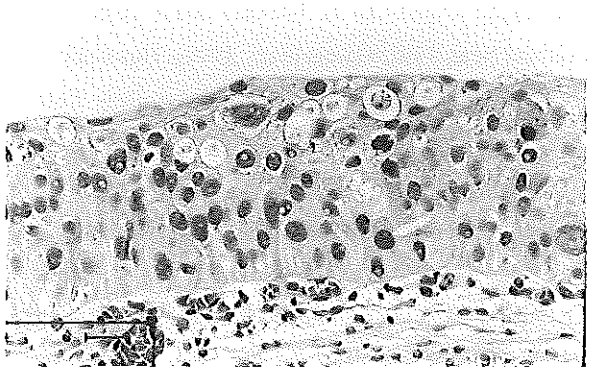

Figure 3: Carcinoma in situ of the urinary bladder.

The clinical course of patients with CIS is difficult to predict. Lamm (1992) compiled the literature data on CIS of the urinary bladder and reported an average incidence of progression to muscle invasive disease of 54%. Furthermore, the occurrence of CIS in the flat urothelium adjacent to tumors is correlated with a higher probability of tumor recurrences and/or invasion [Althausen *et al.*, 1976; Weinstein *et al.*, 1985]. On the other hand, a significant minority of patients with isolated CIS will never have progression to invasive disease [Riddle et al., 1975; Farrow *et al.*, 1976]. This strong variation in natural history has lead to the suggestion that CIS of the urinary bladder may, in fact, be a group of diseases with at least two distinct forms, one being aggressive and the other being relatively indolent [Weinstein *et al.*, 1980; Droller and Walsh, 1985].

Although the clinical behavior of CIS is variable, CIS is in general regarded as a precursor lesion for invasive carcinoma. That CIS, nowadays, is regarded as the most likely precursor lesion for invasive carcinoma is based on evidence gathered from mapping studies, follow-up studies and genetic analyses. First evidence for a preexisting CIS phase before the onset of invasive carcinoma came from mapping studies of cystectomy specimens [Riddle *et al.*, 1975; Farrow *et al.*, 1976; Koss *et al.*, 1977b; Utz and Farrow, 1984; Kakizoe *et al.*, 1984] and from follow-up studies [Farrow *et al.*, 1977]. These studies showed that CIS is related in space and in time to carcinoma. Furthermore, mutation and loss of heterozygosity (LOH) analyses have shown that CIS displays the same genetic alterations as invasive TCC; p53

gene mutations and LOH of chromosome 14q are frequent both in CIS and invasive TCC [Spruck *et al.*, 1994; Rosin *et al.*, 1995].

The reported histological and DNA abnormalities in cells of CIS document the malignant potential of CIS, but additional changes/factors are probably required before progression to invasive disease can occur. To date, high risk factors that are associated with the progression of CIS to invasive disease are diffuse growth, prostatic involvement, overexpression of p53 or proliferation-, surface- or tumor-associated markers and loss of normal urothelial antigens [Hudson and Herr, 1995].

Models for the study of implantation and intraepithelial expansion of bladder cancers

In vitro and in vivo models for the study of bladder tumor cell implantation

Implantation of shedded tumor cells is facilitated by the wounding of the urothelium [Soloway and Masters, 1980]. Urothelial injury exposes the basement membrane and its underlying structures, which contain extracellular matrix proteins (ECM), like laminin, collagen type IV, fibronectin and fibrin [Davis and Avots-Aviotin, 1982; Pode *et al.*, 1986]. Therefore, *in vitro* tumor cell adherence assays study the attachment of bladder tumor cells to ECM-precoated culture dishes. Several studies showed that especially fibronectin promoted cellular attachment *in vitro* [Coplen *et al.*, 1991; See *et al.*, 1992; Hyacinthe *et al.*, 1995]. Furthermore, it was shown that the cellular attachment to fibronectin or other ECM-proteins could be inhibited by the use of specific antibodies (fibronectin-, α5- or β1-integrin antibodies), RGD-peptides or various cytotoxic and immunotoxic agents [Pode *et al.*, 1987; Coplen *et al.*, 1991; See *et al.*, 1992; Hyacinthe *et al.*, 1995].

In vivo the implantation of intravesically installed bladder tumor cells was studied after traumatization of the bladder wall by either cauterization, acid treatment or laser irradiation [Soloway and Masters, 1980; See and Chapman, 1987a; See and Chapman, 1987b]. *In vivo* adherence and implantation could be inhibited by intravesical administration of heparin, chemotherapy, or RGD-peptides [See and Chapman, 1987; Pan *et al.*, 1989; Hyacinthe *et al.*, 1995].

In vitro models of intraepithelial neoplasia

Up till now only few genuine models of intraepithelial expansion (IEE) are documented. Previously, Rebel *et al.* described an *in vitro* model in which a highly-proliferative, outgrowing edge of a semiconfluent bladder culture was confronted with a juxtaposed outgrowing sheet of bladder tumor cells [Rebel *et al.*, 1993]. We further adapted this method to a model that was more comparable with the clinical situation (Figure 5). In this improved model of IEE, bladder carcinoma cells are able to implant and may subsequently expand into the normal surrounding urothelium [Bindels *et al.*, 1997; chapter 2]. Major advantages compared to the earlier model of Rebel *et al.* are 1) implantation of bladder tumor cells in standardized lesions, which results in confrontation with a temporarily regenerating, stratified urothelium; and 2) the possibility for accurate quantification of IEE.

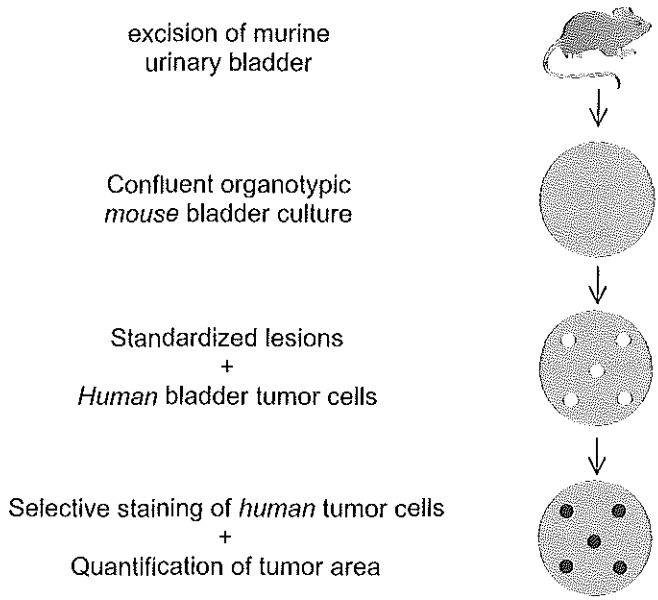

Figure 5: xenogenic cocultivation model for the study of the implantation and subsequent IEE of bladder carcinoma cells.

In another *in vitro* model, culturing of human bladder carcinoma cell lines on top of normal human urothelial stroma resulted in the appearance of an IEE-like phenotype for some of the tested cell lines [Booth *et al.*, 1997].

In vivo models of intraepithelial neoplasia

Several *in vivo* strategies have been described which gave variable outcomes with respect to the formation of intraepithelial neoplasia (IEN). In rodents, spontaneous formation of IEN is not often observed. Exposure of laboratory animals to carcinogenic agents like dibutylnitrosamine (DBN), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), N-methyl-N'-nitrosourea (MNU) N-(4-(5-nitro-2-furyl)-thiazolyl formamide (FANFT) and Bracken Fern can induce CIS of the bladder [Ito *et al.*, 1969; Ohtani *et al.*, 1986; Hicks *et al.*, 1972; Steinberg *et al.*, 1990; Erturk *et al.*, 1967; Bringuier *et al.*, 1995]. However, this procedure has major drawbacks, like a high incidence of papillary and invasive tumors, a long lag time, toxicity to the animal, possible carcinogenicity to other organs, and an often-observed predominance of squamous differentiation [Raghavan et al., 1986; Oyasu, 1995]. This makes these models less attractive as a model of IEN [Hicks and Chowaniec, 1978]. Intravesical

administration of these compounds resulted sometimes in a higher incidence IEN [Samma *et al.*, 1984], although these IEN have a squamous differentiation.

Another approach is xenografting of established bladder carcinoma cell lines in bladders of immunodeficient hosts [Ahlering *et al.*, 1987; Oshinsky *et al.*, 1995; Harabayashi *et al.*, 1999]. Originally, these studies showed a low yield, because the take of bladder tumor cells in intact bladders is largely prevented by an intact bladder surface [Soloway and Masters, 1980]. Therefore, subsequent investigators pretreated the bladder by instillation of chemical agents or induced local trauma to promote tumor take [See and Chapman, 1987]. However, a major limitation of these procedures is the chance of deep penetration of the bladder wall thereby facilitating tumor invasion [Ahlering, 1987]. To prevent this often-observed rupture of the basement membrane and its underlying tissues, we designed an alternative *in vivo* model of IEN (Figure 6; Chapter 6). Overdistension of the urinary bladder of SCID (Severe Combined Immunodeficiency Syndrome) mice resulted in partial removal of the urothelium. Immediately after partial denudation, suspensions of TCC cells were injected into the mouse bladder allowing the formation of IEN.

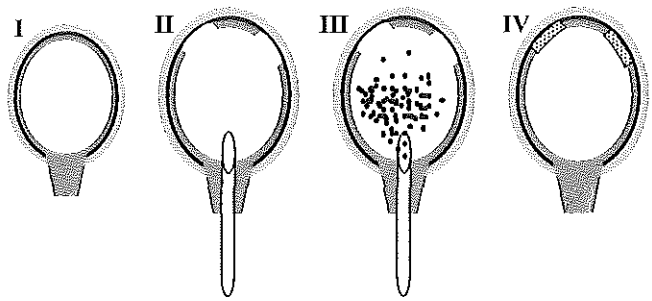

Figure 6: Xenogenic in vivo model for IEN. A normal mouse bladder (I) is overdistended by instillation of saline, resulting in partial removal of the urothelium, without disruption of the basement membrane and its underlying structure (II). Inoculation of bladder carcinoma cells (III). Formation of IEN (IV). [Dark gray: urothelium; Light gray: stroma; Black: basement membrane; Dotted: IEN].

Advanced molecular biological techniques, like the generation of knockout or transgenic mice can be used to study the effect of specific genes on tumorigenesis. However, a major disadvantage of these methods is the often observed lack for the unambiguous determination of gene function in specific tissues, like the bladder. Usage of an urothelium-specific gene construct can circumvent this problem. Recently, Zhang *et al.* used the organ-specific expression of *Uroplakin II* to induce bladder carcinomas [Zhang *et al.*, 1999]. They report that mice bearing a low copy number of an Uroplakin II-SV40T transgene developed

carcinoma *in situ*, whereas mice bearing a high copy number developed CIS, invasive tumors and metastases. In the future, this Uroplakin II- or any other bladder-specific promoter can be used in various site-specific recombination systems to generate tissue-specific knockout mice [Kilby *et al.*, 1993]. A major drawback of the latter approach is that all urothelial cells are targeted and harbor the specific genetical change. Thus, in this model the effect of surrounding normal urothelium on the formation and subsequent IEE of CIS can not be studied. Furthermore, although it is known that the SV40T oncogene can inactivate p53 and retinoblastoma protein (Bryan and Reddel, 1994), its use will probably circumvent the need for other molecular changes of bladder tumorigenesis. In this manner, other relevant genetical changes of bladder cancer will not be identified.

Determinants of intraepithelial expansion

At present, only few determinants of IEE are known. Theoretically, IEE will be determined by the intrinsic features of the tumor cells and the influence of the host environment on the tumor cells (see also chapter 7). In the latter case, IEE will probably be affected by the production of paracine growth factors, deposition of ECM-proteins and cell-cell communication with the surrounding normal urothelium. In a previous *in vitro* model, we have shown that ECM-proteins and growth factors indeed influence the intraepithelial spreading of T24 cells [Rebel *et al.*, 1995]. This could be attributed to a modulation of the normal urothelium rather than a direct effect of the ECM-proteins or growth factors on the tumor cells.

In our *in vitro* model, IEE is the outcome of the balance between the expansion of bladder tumor cells and the regenerative capacity of the surrounding normal urothelium. Shifting this balance towards the regenerative capacity of the normal urothelium will probably also alter IEE. Investigations into the effects of growth factors on urothelial woundhealing in general (Chapter 3) and its effects on cocultivations in particular will contribute to our knowledge of IEE. Therefore, we studied whether stimulation of the regenerative capacity by epidermal growth factor (EGF) could alter the implantation and subsequent IEE of bladder tumor cells (Chapter 2).

Initial studies identified E-cadherin as a factor intrinsic to the bladder tumor cells, that could influence IEE. These studies showed that E-cadherin expression determined the mode of replacement of normal urothelium by human bladder carcinoma cells [Rebel *et al.*, 1994a]. In this thesis, the specific role of E-cadherin in IEE was investigated in more detail (Chapter 4). In the next paragraphs, the general role of EGF and E-cadherin in bladder cancer is discussed in more detail.

Epidermal growth factor (EGF)

The EGF-family consists of a number of related proteins, like TGFα, EGF, amphiregulin, epiregulin, heparin-binding EGF-like growth factor and heregulins [Alroy and Yarden, 1997]. These growth factors exert their effect by binding to specific transmembrane receptors. The EGF-like growth factors bind to erbB-family of tyrosine kinase receptors. The erbB-family is composed of four receptors: erbB-1 (also called EGFR or HER1), erbB-2 (also called HER2 or Neu), erbB3 (or HER3) and erbB4 (or HER4). ErbB1 is the primary receptor for EGF, TGFα

and amphiregulin, whereas erbB3 and erbB4 are the actual specific receptors for heregulins [Carraway and Cantley, 1994].

Several studies emphasize the important role of members of the EGF-family in the normal or abnormal growth of bladder epithelium [Messing *et al.*, 1987]. Furthermore, EGF is implicated in wound healing of the bladder [De Boer *et al.*, 1994; Rebel *et al.*, 1994b; Chapter 3]. Expression of EGF, TGFα and amphiregulin was found in normal human urothelium [Kimball *et al.*, 1984; Cilento *et al.*, 1994] and EGF or TGFα was detected in the urine of respectively healthy individuals [Messing *et al.*, 1987] and bladder cancer patients [Kimball *et al.*, 1984]. In normal human urothelium, EGFR-expression is confined to the basal cell layer, but in bladder carcinomas its expression is seen in all cell layers [Messing *et al.*, 1987]. Also the expression of other members of EGFR-family is increased in TCC [Imai *et al.*, 1995; Rajkumar *et al.*, 1996]. Functional studies showed that EGF, TGFα and amphiregulin could stimulate proliferation and/or migration of normal urothelial cells or bladder carcinoma cells [Rebel *et al.*, 1994b; De Boer *et al.*, 1996; De Boer *et al.*, 1997].

The E-cadherin-catenin complex

Cadherins are a family of transmembrane glycoproteins that mediate Ca^{2+}-dependent intercellular adhesion [Takeichi, 1990]. This family includes the "classical" cadherins, desmosomal cadherins, protocadherins, and products of genes like *c-ret* and *Drosophilia Fat* [Takeichi, 1993]. Cadherins play a well-known role in cell recognition and cell sorting during development [Takeichi, 1988; Takeichi, 1991]. In adult organisms they continue to be expressed in solid tissues. The epithelial form of "classical" cadherins is called E-cadherin. In epithelium, E-cadherin is necessary for the establishment of tissue integrity and polarity. E-cadherin is localized in the adherens junctions and lateral cell surfaces of epithelial tissues and here it forms homophilic cell-cell adhesion complexes. Critical for the function of E-cadherin is its interaction with the catenins [Yap *et al.*, 1997]. The catenins link the E-cadherin molecule to the actin cytoskeleton (Figure 7). α-Catenin possesses an actin-binding activity and therefore probably functions by linking E-cadherin to the actin cytoskeleton [Rimm *et al.*, 1995]. Normally, β-catenin is involved in the linkage of the cytoplasmic tail of E-cadherin to α-catenin. However, in experimental settings it was shown that β-catenin is dispensable for cadherin mediated cell adhesion, as long as α-catenin is fused directly to cytoplasmic tail of cadherin [Nagafuchi *et al.*, 1994]. Together with observations that tyrosine phosporylation of

β-catenin is correlated with diminished cell adhesion [Kinch *et al.*, 1995], this suggests that β-catenin acts as a regulator-site of the cadherin-catenin complex [Gumbiner, 1996].

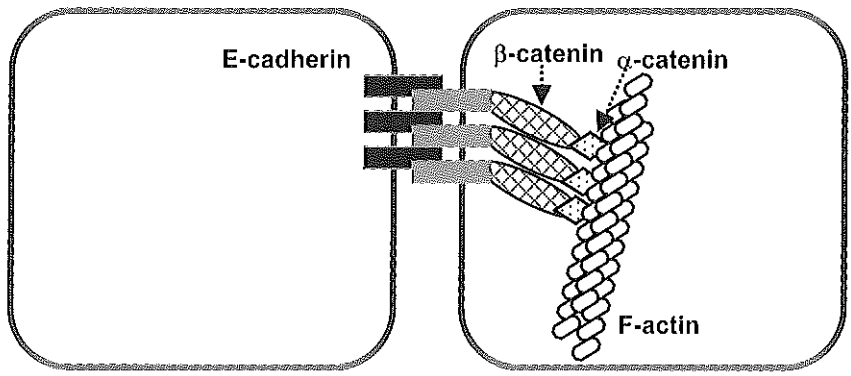

Figure 7: The E-cadherin-catenin complex.

Besides its role in cell-cell adhesion, β-catenin also participates in the WNT-mediated signal transduction pathway [Christofori and Semb, 1999]. Normally, cytoplasmic β-catenin is rapidly phosphorylated by the (APC)-GSK-3β multiprotein complex and subsequently degraded. Cytoplasmic β-catenin can accumulate if this degradation pathway is not functional and the cytoplasmatic tails of E-cadherin are either saturated or defective. In that case, β-catenin can translocate to the nucleus where a β-catenin/TCF transcription complex is formed, which can activate the expression of target genes [Nollet *et al.*, 1999]. Although, γ-catenin can substitute for β-catenin in the cadherin-catenin complex, its physiological relevance is still unknown [Hülsken *et al.*, 1994].

Loss of cell-cell adhesion is implicated in the development and progression of human cancers [Hirohashi, 1998]. Experimental studies have shown the involvement of E-cadherin in the invasive process. Initial *in vitro* studies showed that invasiveness into collagen gel or chicken heart tissue was inversely correlated with E-cadherin expression [Behrens *et al.*, 1989; Frixen et al., 1991]. Moreover, transfection of E-cadherin cDNA into highly invasive, E-cadherin negative cell lines resulted in a reversal of the invasive phenotype [Vleminckx *et al.*, 1991]. Also loss or impaired function of catenins is involved in loss of cell adhesion and the induction of tumor cell invasion *in vitro* [Oyama *et al.*, 1994; Vermeulen *et al.*, 1995]. Clinical studies revealed that the adhesion function of the E-cadherin-catenin complex is lost during the development of various human epithelial cancers [Birchmeier and Behrens, 1994].

The role of E-cadherin-catenin complex in bladder cancer

In normal urothelium E-cadherin is homogeneously expressed at the cell-cell borders. Immunohistochemical studies on the expression of E-cadherin in histopathological material demonstrated that aberrant E-cadherin expression correlates with lack of differentiation, muscle invasion and metastasis [Syrigos et al., 1999]. Loss of E-cadherin also correlated with a decreased recurrence-free and overall survival [Bringuier et al., 1993; Syrigos et al., 1995; Liponnen and Eskelinen, 1995; Shimazui et al., 1996]. In addition, abnormal expression of catenins or $p120^{cas}$ was also associated with tumor grade, stage, and poor prognosis [Shimazui, 1993; Syrigos et al., 1998a; Syrigos et al., 1998b]. None of the previous reports performed detailed studies on the E-cadherin immunoreactivity of CIS of the bladder.

Frixen *et al.* (1991) noted that two *in vitro* non-invasive bladder carcinoma cell lines, RT112 and RT4, expressed E-cadherin, whereas an *in vitro* invasive cell line, EJ28, did not. Recently, Giroldi *et al.* (1999) studied the expression levels of various members of the classical cadherins in a panel of 17 bladder carcinoma cell lines. They found that in cell lines, which lost E-cadherin, additional changes in catenins occurred and that N-cadherin became predominantly expressed in these cell lines [Giroldi *et al.*, 1999]. Hazan *et al.* proposed that N-cadherin promotes the interaction between tumor cells and stromal cells, thereby facilitating invasion [Hazan *et al.*, 1997]. This suggests that the observed upregulation of N-cadherin in bladder carcinoma cell lines could be involved in bladder tumor invasion.

Integrin-Extracellular matrix interactions

It has been shown that intraepithelial lesions expand along the basement membrane, thereby undermining the normal urothelium [Murphy, 1994]. The basement membrane of epithelia mainly consists of ECM proteins, like collagen type IV and laminin, and proteoglycans. The integrins, belonging to the family of cell adhesion molecules (CAMs), are the receptors for these ECM-proteins [Hynes, 1992; Giancotti and Mainiero, 1994]. The integrin family is composed of 15 α and 8 β subunits that are contained in to some 25 different $\alpha\beta$ heterodimeric combinations on cell surfaces. Nowadays, it is well recognized that integrins are involved in motility, invasion, signal transduction, proliferation, apoptosis and angiogenesis [Varner and Cheresh, 1996; Keely *et al.*, 1998; Sanders *et al.*, 1998; Aplin *et al.*, 1999, Ruoslahti, 1999].

Immunohistochemical analysis showed that invasive bladder carcinomas have a reduced expression of α2, α3 integrin and an increased expression of α5 integrin (Liebert *et al.*, 1994a; Saito *et al.*, 1996]. Furthermore, Liebert *et al.* (1994b) found that bladder carcinomas exhibited a loss of co-localization between α6β4 integrin and collagen VII. The α6β4 integrin is a component of the hemidesmosomal complex, which anchors the basal epithelial cells to the basement membrane [Sonnenberg *et al.*, 1991]. Therefore, loss of α6β4 integrin in bladder carcinoma cells will increase their motility. More recent, Harabayashi *et al.* (1999) showed that reduction of β4 stimulated intraepithelial spreading of CIS on laminin.

Scope of the thesis

A well-known, major problem in the management of superficial TCC is the high frequency of bladder tumor recurrences. Assuming that these recurrences belong to the same clone as the primary tumor, implantation and intraepithelial expansion (IEE) of bladder tumor cells are the most likely mechanisms to explain these recurrences. Clinical and experimental studies confirmed that this could be the case in most instances. However, the factors that are involved in both mechanisms are only partially understood.

Therefore, the aim of the studies presented in this thesis was the development of *in vitro* and *in vivo* models, that could be useful in the study of 1) the implantation and 2) IEE of bladder tumor cells. Subsequently, these models can be used to define factors that are involved in both processes. Both the intrinsic properties of the tumor cells and the influence of the surrounding urothelium can be studied in these models.

In chapter 2, a new *in vitro* cocultivation model is described, in which the implantation and subsequent IEE of bladder tumor cells could be studied. A major advantage of this model is the possibility for accurate measurements of tumor areas as it expands into the normal surrounding urothelium (IEE). In the same chapter, we investigated whether stimulation of the regenerative capacity (by EGF) of the traumatized urothelium could counteract TCC recurrences. In chapter 3, the role of EGF-EGFR family during the regeneration of normal urothelium was studied in more detail. Using the previously described cocultivation model the IEE capacity of a panel of six different TCC cell lines, with various levels of E-cadherin expression was studied (chapter 4). Searching for differences/similarities between IEE and invasion, we characterized the invasive properties of our panel of TCC cell lines and correlated those with E-cadherin expression of the cell lines (chapter 5). To confirm the *in vitro* findings on IEE, *in vivo* models are required. Chapter 6 describes the establishment of an in vivo model of CIS (an intraepithelial neoplasia).

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Chapter 2

IN VITRO MODULATION OF IMPLANTATION AND INTRAEPITHELIAL EXPANSION OF BLADDER TUMOR CELLS BY EPIDERMAL GROWTH FACTOR

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Abstract

A major problem in the management of bladder cancer is the high risk for recurrence of bladder tumors after transurethral resection. This has generally been attributed to the attachment and subsequent expansion of exfoliated tumor cells to the traumatized bladder wall. An *in vitro* cocultivation model was used to study the implantation and growth of human tumor cells in traumatized murine urothelium. Furthermore, we investigated in a time course experiment whether stimulation of the regenerative activity of the normal urothelium by a growth factor could affect implantation and subsequent growth of bladder tumor cells.

After inoculation on injured confluent cultures of murine urothelium, human T24 and SD bladder carcinoma cells preferentially attached to the denuded areas. SD cells expanded into the normal urothelium as a sharply demarcated tumor, while T24 cells infiltrated as single cells. Treatment of the primary urothelium with epidermal growth factor (EGF) stimulated the proliferation of the primary urothelium and reduced the implantation and growth of T24 considerably. EGF reduced the implantation of the SD tumor cells but could not prevent the further expansion at the expense of surrounding normal urothelium. Since EGF had no effect on migration or proliferation of SD or T24 cells, its modulation of expansive growth is most probably due to an increase in the regeneration of normal urothelium.

This study suggests that recurrence of transitional cell carcinomas might in some instances be inhibited by stimulation of the regeneration of traumatized urothelium. The reported *in vitro* cocultivation model may be useful for studying additional factors involved in intraepithelial expansion of carcinoma cells.

Introduction

Human bladder cancer is the fifth most common cancer in males in western society [1]. Of the patients presenting with human bladder cancer, approximately 65% have superficial transitional cell carcinomas (TCC). The remaining patients have invasive tumors penetrating the muscularis propria and underlying tissue. The patients with superficial bladder cancer have a considerable risk of tumor recurrence (up to 70%) and progression [2].

Two hypotheses have been put forward to explain TCC recurrences. First, on the basis of the field cancerization theory, some authors suggested that each recurrent tumor had arisen *de novo* from precancerous flat urothelium [3]. Second, these recurrent tumors may be the progeny of a single transformed cell, implying their derivation from residual tumor cells. The monoclonality of multiple synchronous or metachronous bladder tumors is strongly in support of the latter hypothesis [4-6].

Several clinical and experimental data indicate that bladder tumor recurrences could be the consequence of shedding and subsequent reattachment of tumor cells to (traumatized) areas in the urothelium [7-10]. In addition, after surgery, residual tumor cells may cause tumor recurrences at the original site. Thus, two possible mechanisms may underlie recurrences of TCC: (1) intraluminal shedding and seeding of TCC cells on traumatized or intact urothelium and/or (2) lateral expansion of residual TCC cells into the normal urothelium. An *in vitro* model is required to study the cell biological mechanisms underlying these tumor recurrences.

Previously we have shown that murine explant cultures on porous membranes mimic the *in vivo* situation; the cultured urothelium shows multilayering and differentiation into umbrella cells [11]. In an *in vitro* cocultivation model the influence of ECM and growth factors on the pattern of infiltration of T24 in surrounding normal urothelium was examined. Exposure of the cultures to acidic fibroblastic growth factor (FGF-1) and laminin led to a significant increase in the number of T24 cells infiltrating the normal urothelium, whereas epidermal growth factor (EGF) and collagen type I and IV counteracted the distance and number of infiltrating T24 cells [12].

Here, we describe a new xenogenic cocultivation model, which allows more accurate measurements of tumor areas as it expands into the normal urothelium. This newly developed model was used to study the implantation and expansion of two human bladder carcinoma cell lines: SD, an E-cadherin expressing bladder tumor cell line derived from a well differentiated

bladder tumor and T24, an E-cadherin negative bladder tumor cell line derived from a poorly differentiated TCC [13, 14].

Although under normal circumstances the cell turnover of urothelium is very low, urothelial damage results in a very rapid regeneration [15-17]. In an organotypic mouse bladder culture model the regeneration rate of normal urothelium can be stimulated by EGF [18, 19]. Addition of EGF, prior to the induction of the injuries, was used to modulate the implantation and expansion of human bladder carcinoma cells. In the case of the T24 cell line addition of EGF almost completely inhibited the outgrowth of tumor cells. This suggests that in some instances recurrence of TCC may be counteracted by intraluminal growth factor treatment of traumatized urothelium.

Materials and methods

Primary cultures of murine urothelium

Murine urinary bladders were dissected from 6- to 8 week-old female C3H/He mice and cut into halves. The mucosa was stripped from its underlying muscle layer and spread out on a collagen type IV coated cyclopore membrane (Falcon culture-insert, Becton Dickinson, Etten-leur, Netherlands) with the submucosa facing the culture support. Collagen coating was performed by incubation of the cyclopore membranes with 25 μg/ml human collagen type IV (Fluka, Oud Beijerland, Netherlands) as described previously [12]. Standard culture medium consisted of a 1:1 mixture of DMEM and HAM's F10, supplemented with 10% heat-inactivated FCS, 10 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenite, 50 nM hydrocortisone, 10 μM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The explant cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and grown to confluence.

In some experiments, 20 ng/ml EGF (Sigma, St. Louis, USA) was added to the culturing medium 4 days before confluence (±75% confluent) until the cultures were terminated. Previous studies had shown that this dose of EGF optimally stimulates the proliferation of mouse urethelial cultures. The medium supplemented with EGF was changed every day.

Traumatization of normal mouse urothelium and inoculation of bladder tumor cells

Injuries were made 2-3 days after confluence. Five circular areas (four peripheral, one central lesion) were denuded with a biopsy punch (Stiefel, Offenbach am Main, Germany; 3 mm diameter). The urothelium in the injured area was scraped away with a glass policeman. The advantage of this procedure is that an imprint of the biopsy punch remains visible on the membrane and that the injury can be made in a standardized fashion, allowing the monitoring of the boundaries of the circular injured area during the experiments. After injury the cultures were washed twice with PBS followed by inoculation of 10^5 tumor cells in 1.5 ml normal or EGF-supplemented medium on the murine urothelial explant cultures. The bladder tumor cells were allowed to attach to the injured areas in the explant cultures for 24 h; next, the nonadherent cells were washed away with PBS and the cultures were either terminated or continued for another 4, 7, or 14 days in normal medium or EGF-supplemented medium. Each experiment was performed in duplicate and all experiments were repeated twice.

Determination of proliferative activity

Two hours before termination of the culture, 40 µg/ml BrdU in normal medium was added, and the cultures were rinsed twice with PBS and fixed in 70% ethanol for at least 24 hours. Proliferative activity in normal and injured control cultures, without tumor cells, on Days 0, 1, 4, 7, and 14 was assessed by determination of the labeling index (L.I.) and the nuclear density. Four areas of 0.15 mm^2 around each wounded area were counted. The L.I. is defined as the relative number of BrdU positively stained nuclei in an area of 0.15 mm^2. The nuclear density is the total number of nuclei in an area of 0.15 mm^2.

Immunohistochemistry

In the case of the BrdU staining, ethanol fixed cultures were pretreated with 2 M HCl and Borax buffer, pH 8.5 [20]. Nonspecific binding was blocked with 10% normal goat serum in 1% BSA/PBS. Monoclonal antibody RCK108 (Eurodiagnostica, Apeldoorn, Netherlands) is specific for human cytokeratin 19 and has no cross-reactivity with mouse urothelium. Almost 100% of T24 and SD cells are labeled with RCK108. This feature permits the selective identification of human bladder cancer cells in this cocultivation model [21].

The primary antibodies RCK108 and antibodies against BrdU (Gift of Prof. F. Ramaekers, University of Maastricht, Netherlands) were visualized in a two-step peroxidase staining method. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (DAKO, Glostrup, Denmark). 3,3-diaminobenzidine tetrahydrochloride (DAB) (Fluka), diluted in PBS (2 mg/ml) served as chromogen using 0.03% H_2O_2 as substrate. Control cultures were counterstained in hematoxylin, dehydrated, and mounted in Euparal (Chroma, Köngen, Germany).

Image analysis

The RCK108-immunostained area covered with T24 or SD tumor cells within the normal urothelium was visualized with a Hitachi CCTV camera and quantified using KS400 image analysis software (Kontron Elektronik, Eching, Germany; KS400, Version 1.2). RCK108-stained areas were expressed in square millimeters. In the case of the RCK108-immunostained T24 cells, a differentiated silver intensification procedure was used to enhance the DAB signal [22].

[^{3}H]thymidine incorporation

To assess the possible growth stimulating effect of EGF on both tumor cell lines, a cell kinetic study was performed. T24 or SD cells grown in 75-cm^2 culture flasks were washed in serum-free medium and trypsinized; 10^5 cells were seeded in collagen type IV (25 µg/ml) coated 96-multi-well dishes (Nunc, Roskilde, Denmark) and were cultured for 96 h. After 24 h of incubation in SF medium, various concentrations of EGF diluted in either SF medium or standard medium were added. Serum-free medium was similar to the standard medium, but without FCS, and supplemented with 0.1 % BSA, 4 µM spermine, 4 µM spermidine, 0.1 mM ethanolamine and 1 µM putrescine. Proliferative activity was determined at 24, 48, 72, and 96 h. During the final 16 h of culture, cells were incubated with 0.5 µCi [^{3}H]thymidine (Amersham,'s-Hertogenbosch, Netherlands) per well and subsequently trypsinized and harvested. The incorporated [^{3}H]thymidine was counted using a BetaPlate scintillation counter (LKB-Pharmacia, Woerden, Netherlands), and expressed as counts per minutes.

Cell motility assay

Chemotaxis was assayed using 48-well microchemotaxis Boyden chambers with 8 µm pore size polycarbonate Nucleopore filters [23]. T24 and SD cells were passaged by trypsinization and plated at a density of 1x10^6 cells/10 ml in 75-cm^2 culture flasks 1 day prior to the assay. Cells were harvested immediately before the assay with 2 mM EDTA. EGF (20 ng/ml) tested as a chemoattractant was diluted in DMEM and added to the lower compartment, while the upper compartment was filled with DMEM/0.1% BSA containing 25,000 cells. Human fibronectin (100 ug/ml; Sigma) was used as a positive control. Cells were allowed to migrate for 4 h in a CO_2 incubator at 37°C. At the end of the assay, filters were removed, fixed in cold methanol, treated with RNase and stained with hematoxylin. The number of migrated cells was counted in a random area of 0.375 mm^2 per well.

Statistical methods

A Mann-Whitney U test was used to determine the statistical significance of the data concerning the attachment of bladder tumor cells on intact (EGF-stimulated) urothelium.

Results

Regenerative capacity of normal or EGF-treated urothelium

The injured areas (both peripheral and central) in the nontreated confluent murine urothelial cultures were covered within 24 h (Figs. 1A, 1B and 1C). Since previous studies had shown that differences in proliferation existed between the central and the peripheral part of the outgrowth of semiconfluent murine explant cultures [18], regenerative capacities of peripheral and central lesions were evaluated separately.

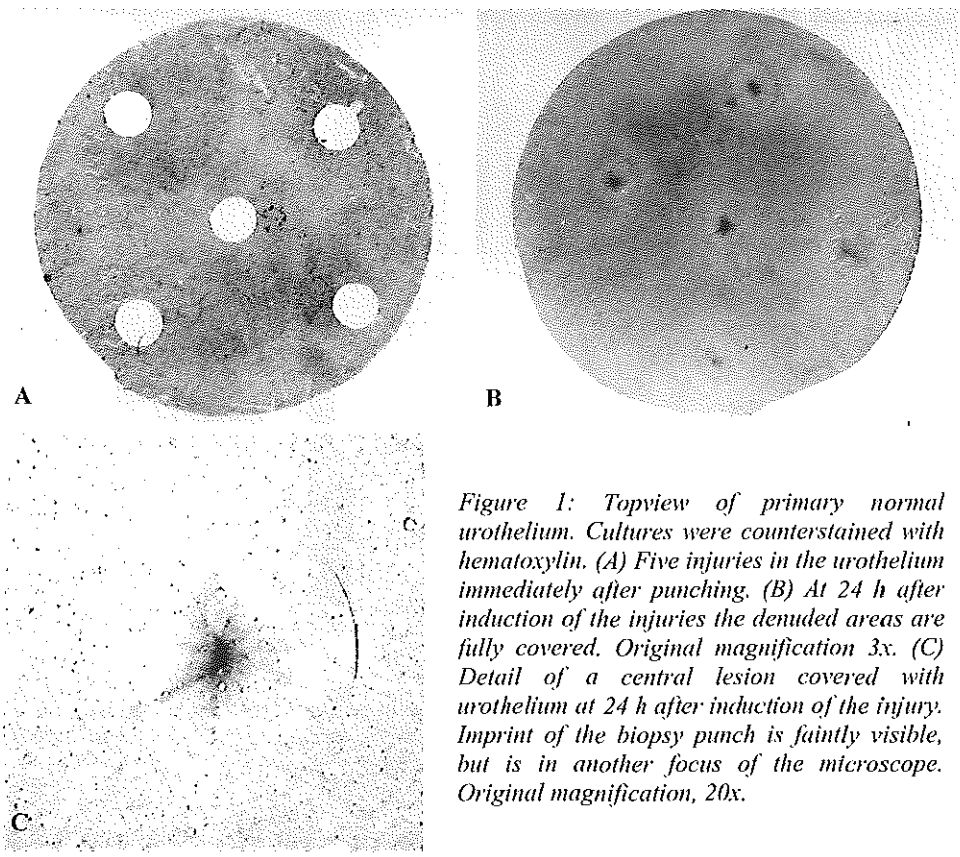

Figure 1: Topview of primary normal urothelium. Cultures were counterstained with hematoxylin. (A) Five injuries in the urothelium immediately after punching. (B) At 24 h after induction of the injuries the denuded areas are fully covered. Original magnification 3x. (C) Detail of a central lesion covered with urothelium at 24 h after induction of the injury. Imprint of the biopsy punch is faintly visible, but is in another focus of the microscope. Original magnification, 20x.

Other than a small but consistent decrease of the nuclear density at 24 h after injury around both the peripheral and the central lesions in untreated cultures, no further change in the nuclear density was seen at different time points (Fig. 2A). Apparently the overall status of the control urothelium did not change during the 14 day culture period after confluence. The

injuries in the further untreated cultures did not lead to a significantly increased proliferation (Fig. 2B). Within the regenerated areas BrdU-positive cells were rarely present. Exposure of noninjured and injured confluent urothelial cultures to EGF stimulated the proliferation and led to an increase in cellularity of the urothelium compared to that of urothelium not exposed to EGF (Figs. 2A and 2B). Again, BrdU positive cells were rarely present within the regenerated areas.

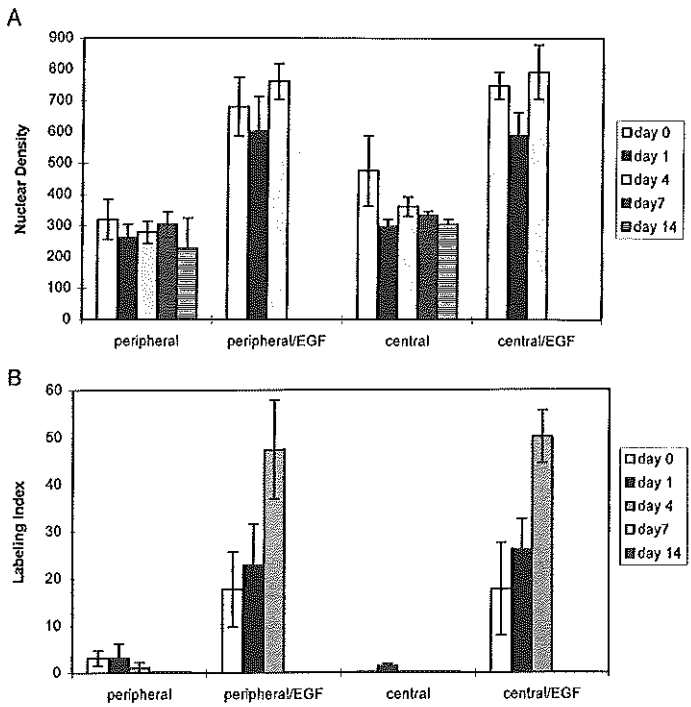

Figure 2: Nuclear density and BrdU incorporation in normal or EGF-treated injured primary urothelium (n=6, mean ± SD). Nuclear density and labeling index were not determined for the EGF-treated injured primary cultures on Days 7 and 14.

In summary, the peripheral and central injured areas did not reveal a difference in nuclear density or labeling index, except for the somewhat higher nuclear density on Day 0 around central lesions. The results indicate that in untreated cultures the closure of the injuries, without a marked increase in proliferation, can largely be attributed to migration of cells into the injured area. The characteristic decrease in nuclear density at 24 h after injury is in favor of this explanation.

Expansion of T24 or SD on untreated and EGF-treated injured urothelium

After seeding of 10^5 T24 cells on top of the confluent urothelial cultures with five circular injured areas, these cells exclusively attached to the five denuded areas. Within 24 h the whole lesion was covered with T24 cells and regenerating urothelium. Strikingly, the (RCK108 positive) T24 cells continued to infiltrate the normal murine urothelium as single cells (Fig. 3A). Morphometric analysis of the area occupied by T24 cells in peripheral or central lesions showed a slow and gradual increase in the area occupied by T24 tumor cells (Figs. 4A and 4B).

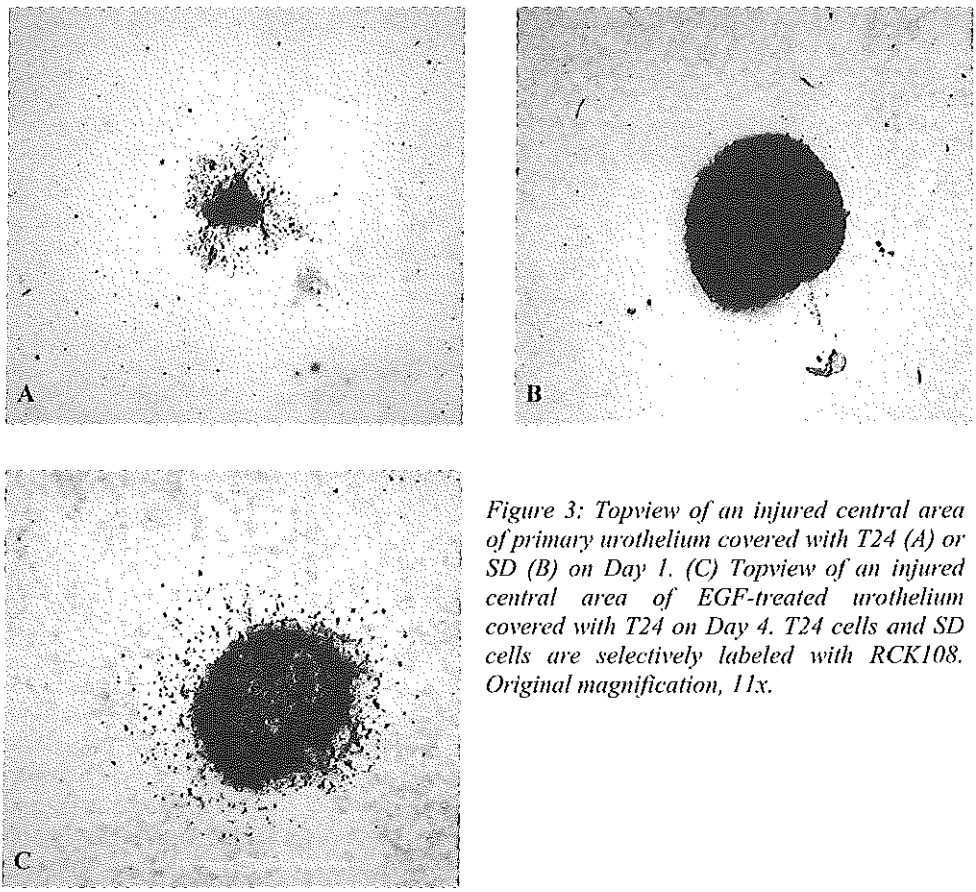

Figure 3: Topview of an injured central area of primary urothelium covered with T24 (A) or SD (B) on Day 1. (C) Topview of an injured central area of EGF-treated urothelium covered with T24 on Day 4. T24 cells and SD cells are selectively labeled with RCK108. Original magnification, 11x.

SD cells attached primarily to the damaged area of the urothelium, but small colonies of SD cells also adhered to the intact urothelium. Twenty-four hours after inoculation, the damaged area was filled with SD cells bordered by regenerating urothelium. A sharp demarcation was seen between the normal urothelium and the SD cells (Fig. 3B). No single tumor cells penetrating the surrounding normal urothelium were observed. Morphometric analysis of the area filled with SD cells in peripheral or central lesions showed a marked increase in time of the area filled with SD cells (Figs. 4C and 4D). The increase in SD occupied area was more pronounced in the peripheral lesions than in the central lesions.

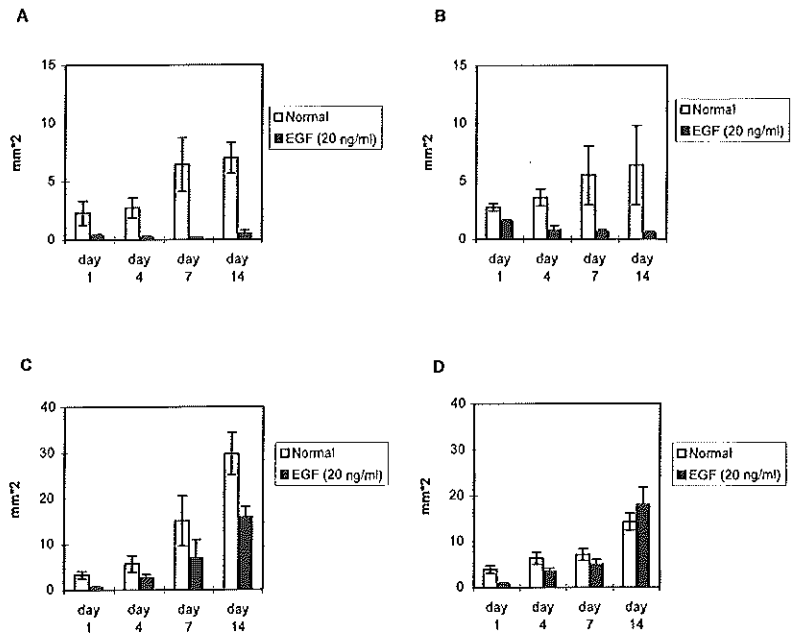

Figure 4: Graphs display the areas occupied by T24 cells in peripheral (A) or central (B) lesions of the urothelium measured on different time points in normal or EGF-stimulated cultures. Areas occupied by SD cells in peripheral (C) or central (D) lesions of the urothelium measured on different time points in normal or EGF-stimulated cultures. Data are expressed as mean ± standard deviation (n=6 cultures). Note the difference in y-axis between A and B and C and D

Treatment with EGF significantly reduced the area occupied by T24 cells in both the peripheral and the central lesions throughout the whole course of the experiment (Figs. 3C, 4A and 4B). In comparison with the nontreated cultures EGF pretreatment diminished the area of initial implantation of SD, but did not block the further expansion of SD (Figs. 4C and

4D). The reduction of the implantation of both T24 and SD tumor cells can be explained by the stimulation of wound regeneration by EGF.

Attachment of T24 and SD cells on intact, EGF-stimulated urothelium

It was previously shown that E-cadherin-negative human bladder tumor cells are not able to attach to intact murine urothelium, in contrast to E-cadherin-positive human bladder tumor cells [9]. Since EGF stimulates multilayering but inhibits differentiation into umbrella cells of the bladder cultures [18, 19], we wished to study whether T24 can attach to confluent, EGF-stimulated urothelium. After inoculation of a suspension of 10^5 T24 cells/1.5 ml no attachment to intact untreated or EGF-pretreated confluent murine urothelium was observed.

EGF stimulation had no significant effect on the implantation of SD cells; 24 h after addition of the cells 25.2 ± 14.9 [mean ± SEM (n=5)] colonies of SD were observed to be attached to the nontreated cultures compared to 10.8 ± 5.3 [mean ±SEM (n=5)] colonies of SD to the EGF-treated cultures (Mann-Whitney test; $P > 0.1$).

Effect of EGF on proliferation and migration of T24 or SD cells

To be able to distinguish between the observed direct effects of EGF on the regenerating normal urothelium and possible additional effects on the tumor cells, we measured the effect of EGF on proliferation and/or migration of T24 and SD cells. When EGF was added to T24 or SD cells growing in either serum-free medium or standard medium, we could not observe any effects on the incorporation of [^{3}H]thymidine (results not shown). Migration of the tumor cells was measured in a modified Boyden chamber chemotaxis assay. Fibronectin (serving as a positive control substance) stimulated migration of both T24 and SD bladder carcinoma cells: 353.7±6.5 T24 cells and 107.0±29.8 SD cells had migrated across the membrane. In contrast, EGF did not induce migration of T24 or SD carcinoma cells: in the presence and absence of EGF 2.0±1.0 and 3.0 ±1.0 T24, respectively, cells were counted on the opposite site of the filter. In the case of SD, the figures were 1.0±1.0 and 2.3±1.0 cells, respectively.

Discussion

With the establishment of the xenogenic *in vitro* cocultivation model reported here we were able to study the implantation of bladder carcinoma cells on intact and injured urothelium, and their subsequent expansion. The murine explant cultures mimic the *in vivo* situation and show the presence of multiple layers and differentiation into umbrella cells. A further advantage of this model is that it can easily be manipulated by exposition to growth factors and ECM proteins [18, 19]. In a previous cocultivation model of our laboratory tumor cells were confronted with the outgrowing edge of a nonconfluent primary explant culture [12, 21]. The periphery of such an outgrowing culture consists of only one cell layer, whereas in the *in vitro* model reported here the edge of the denuded area is multilayered. A major advantage of the described approach is the possibility of directly visualizing the expansion of tumor cells in relation to the surrounding urothelium. The imprint made with the punch during traumatization serves as an excellent marker of the originally denuded area.

After infliction of damage to the confluent urothelial cultures, a rapid regeneration occurred and the lesions became covered within 24 h. This rapid regeneration was not associated with a significant increase in proliferative activity of the urothelium at the edges of the injured areas. This suggests that the regeneration must be due to migration of cells into the injured area rather than proliferation. The characteristic and consistent decrease in nuclear density at the edges surrounding the injured areas at 24 h is in favor of this explanation.

The high recurrence rate of bladder cancer after transurethral resection of the primary tumor has been attributed to the selective implantation of shedded tumor cells on traumatized bladder mucosa [24].

The selective colonization of the injured areas after inoculation of T24 and SD cells parallels the clinical observations. However, small clusters of SD cells, but not T24 cells, were also able to attach and colonize intact urothelium. In a previous study we demonstrated the role of E-cadherin expression in this process [9]. Either a homotypic E-cadherin binding of the SD cells with primary urothelium or the E-cadherin mediated formation of SD aggregates is required for the implantation on intact urothelium. Stimulation of primary urothelium with EGF did not significantly influence the implantation of SD cells. Since EGF stimulation results in murine explant cultures largely devoid of terminally differentiated umbrella cells, this latter cell type does not seem to play a specific role in the attachment of

SD to intact urothelium [18, 19]. Further studies are needed to identify the specific molecular mechanisms accounting for the colonization of intact urothelium by tumor cells.

Initially, after inoculation of T24 or SD cells the injured area occupied by these cells was in general smaller than the originally denuded area. Competition with regenerating (migrating) primary urothelium is probably the cause for this effect. After implantation of T24 and SD cells to denuded areas in the urothelium, both types of tumor cells were able to expand at the expense of the primary urothelium, but the expansion of SD cells was most pronounced, particularly in the injured areas in the periphery of the explant culture, where nuclear density of surrounding normal urothelium was lowest. Probably in the central denuded areas a greater number of normal urothelial cells could migrate from the margins into the denuded area, offering more competition with inoculated SD or T24 tumor cells.

The implantation and outgrowth of T24 in the *in vitro* cocultivation model was counteracted by EGF treatment. EGF stimulates the proliferation, but also the regeneration of primary murine and human urothelial cells in an *in vitro* model of bladder regeneration [18, 19]. In other epithelial cell systems it has been shown that EGF can stimulate both cell migration and proliferation [25, 26]. Furthermore, EGF may induce the synthesis of specific extracellular matrix proteins [27]. EGF had, however, no significant effect on the proliferation or migration of the T24 carcinoma cells. This lack of responsiveness of T24 cells to EGF can perhaps be ascribed to the activating Ras-mutation [28] in this cell line, which obliterates the need for EGF receptor activation. Therefore, the most logical explanation for the observed inhibition of the intraepithelial expansion of T24 cells by EGF is an enhanced regeneration of the primary urothelial cultures. EGF treatment did not influence the intraepithelial expansion of SD cells. Since EGF did not affect proliferation or migration of SD cells the capacity for intraepithelial expansion of SD cells seems to be an intrinsic property of these cells, which enables them to compete successfully with the primary urothelium.

Cross sections of cocultivation cultures demonstrated that both T24 cells [21] and SD cells (data not shown) infiltrate underneath the cells of the normal urothelium. This pattern of "undermining" intraepithelial expansion resembles the method of expansion of carcinoma *in situ* at the expense of normal urothelium *in vivo* [29]. A more effective interaction of tumor cell integrins with extracellular matrix proteins of the basement membrane with a higher affinity than the interaction of integrins of normal urothelium may offer a possible explanation for the lateral expansion of carcinoma *in situ* cells. Alterations in integrin expression profiles by transitional cell carcinoma cells may point to such a mechanism [30,

31]. Maybe EGF or other growth factors could alter the expression of integrins on normal or tumor cells, facilitating the intraepithelial expansion of bladder tumor cells. For instance, EGF upregulated cell adhesion and migration of HSC-1 carcinoma cells, keratinocyte migration, and regeneration of human airway epithelium via an effect on the regulation of integrin expression [32-34].

In conclusion, our data suggest that stimulation of the regeneration of the normal urothelium could diminish the implantation of TCC to traumatized areas in the bladder, but not to intact urothelium. Further lateral expansion of implanted tumor cells could be prevented in the case of T24 cells, but not SD cells.

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Chapter 3

FUNCTIONS OF EPIDERMAL GROWTH FACTOR-LIKE GROWTH FACTORS DURING HUMAN UROTHELIAL REEPITHELIALIZATION *IN VITRO* AND THE ROLE OF ERBB2

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Abstract

Transitional epithelium of the urinary bladder can be damaged during e.g. catheterization, overstretching due to obstructed voiding, or partial resection. The subsequent repair process can be stimulated by specific proteins like epidermal growth factor (EGF) and transforming growth factor-α (TGFα). However, little is known about the role of EGF-like growth factors and their respective receptors in human urothelial repair. In this study, we examined effects of EGF, TGFα, amphiregulin and heregulin-α (HRGα) on proliferation, wound closure, and expression of their receptors c-erbB1 to c-erbB4 in primary cultures of human urothelial cells *in vitro*.

Under conditions representing intact urothelium, all EGF-like growth factors but HRGα induced proliferation. TGFα stimulated proliferation up to four times. Amphiregulin increased expression of c-erbB1. Treatment with either TGFα or amphiregulin resulted in higher c-erbB1 activation and c-erbB3 levels. None of the growth factors affected the constitutive expression of c-erbB2 and c-erbB4. In the repair model, both EGF and TGFα stimulated the wound closure most strongly. This was mainly achieved by increased cellular migration. Receptor expression was not affected by exogenous growth factor addition. The role of c-erbB2 in wound healing was further investigated with the use of antisense DNA. Wound closure could be delayed up to 50% by antisense c-erbB2 but not by mismatched or sense oligonucleotides.

Excessive production (e.g. in bladder tumors) or application of EGF, TGFα or amphiregulin, but not HRGα may lead *in vivo* to either hyperplasia or a faster repair of damaged urothelium. These effects seem to be mediated not only via c-erbB1 but also via c-erbB2. Our results suggest that modified members of the EGF-EGFR family are potential targets for future therapies concerning bladder wound healing and malignancy.

Introduction

Normally, the cell turnover of bladder epithelium is very slow [24], but urothelial damage e.g. by catheterization, outlet obstruction, deposition of urinary crystals or partial resection, results in a very rapid regeneration [14]. Urothelial regeneration is a tightly regulated process involving proliferation, migration, differentiation and extracellular matrix production. Peptide growth factors are involved in all of these steps of wound healing. Several studies demonstrated that members of the EGF-family are important mediators of wound healing. The EGF-family consists of a number of related proteins, like TGFα, EGF, amphiregulin, epiregulin, heparin-binding EGF-like growth factor and heregulins [2]. Exogenous application of EGF, TGFα or heregulin stimulates epidermal wound healing *in vivo* [7, 12, 32]. In addition, EGF and TGFα promote the reepithelialization of the injured gastrointestinal tract [29].

The EGF-like growth factors bind to erbB-family of tyrosine kinase receptors. The erbB-family is composed of four receptors: erbB-1 (also called EGFR or HER1), erbB-2 (also called HER2 or Neu), erbB3 (or HER3) and erbB4 (or HER4). ErbB1 is the primary receptor for EGF, TGFα and amphiregulin, whereas erbB3 and erbB4 are the actual specific receptors for heregulins [8]. Although a specific ligand for erbB2 is still unknown, c-erbB2 plays a major coordinatory role in the erbB-family, because it has the ability to form heterodimers with every other erbB-receptor. Also the other members of the erbB-family can form homodimers or heterodimers with each other [2]. Dimerized erbB-receptors concomitantly autophosporylate and thereby become docking sites for proteins bearing SH2 domains, which in turn couple to downstream signaling pathways [22].

The epidermal growth factor-family probably plays an important role in the normal and abnormal growth of bladder epithelium [26]. Expression of EGF, TGFα and amphiregulin was found in normal human urothelium [9, 25] and EGF and TGFα were detected in the urine of healthy individuals [26] and bladder cancer patients, respectively [25]. In normal human urothelium, EGF-receptor expression is confined to the basal cell layer, but in bladder carcinomas the expression is seen in all cell layers [26]. Also the expression of other members of EGFR-family is increased in carcinoma of the bladder [23, 30]. Functional studies showed that EGF, TGFα and amphiregulin could stimulate proliferation and/or migration of normal urothelial cells or bladder carcinoma cells [16, 17, 31]. Although these studies emphasize the importance of the EGF-family in the normal or abnormal growth of the bladder, little is known

about the function of EGF-family members and their erbB-receptors in wound healing of the bladder.

Previously, we have shown that EGF can stimulate reepithelialization in an organotypic culture of mouse urothelium [15, 31]. In the present study, an analogous *in vitro* model of human urothelium, which closely mimics differentiation and multilayering of normal urothelium [16], was used to investigate the role of members of the EGF-family in normal and regenerating urothelium. Results of our study will be beneficial to the field of bladder reconstruction and replacement surgery. Furthermore, knowledge about the function of the EGF/EGFR family in maintenance and repair of normal urothelium will also be relevant for urothelial tumorigenesis, because growth factors and their receptors can serve as modulators of tumor cell biology, thereby influencing tumor growth rate, local invasion and metastasis.

Materials and methods

Chemicals

Biochemicals were obtained as follows: tissue culture additives and EGF (Sigma, St. Louis, USA); human collagen type IV (Fluka, Buchs, Switzerland); amphiregulin and HRGα (R&D Systems, Minneapolis, USA) and TGFα (Boehringer Mannheim, Mannheim, Germany). Previously, effective concentrations of these ligands were determined by construction of dose-respons curves [17]. Antibodies were purchased as follows: anti c-erbB1 (Oncogene Research, Uniondale, USA); anti phosphorylated c-erbB1 (Transduction Laboratories, Lexington, USA); anti c-erbB2 and secondary antibodies (DAKO, Glostrup, Denmark); and anti c-erbB3 and anti c-erbB4 (Santa Cruz Biotechnology, Santa Cruz, USA). The anti-BrdU antibody was kindly donated by Dr. B. Schutte (University of Maastricht, Maastricht, The Netherlands).

Cell Culture

Primary human explant cultures were established as described previously [16]. Briefly, fresh and macroscopically normal appearing ureter specimens were obtained from resection material from patients treated for non-malignant renal diseases. The urothelium was stripped from the submucosa and processed onto cyclopore membranes (Falcon culture inserts, Becton Dickinson) coated with collagen type IV (25 µg/ml). Primary explant cultures were obtained using DMEM/Ham's F12 medium supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenite, 5 nM hydrocortisone, 10 mM HEPES, 2 mM glutamine, 100 IU penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FCS. This medium is referred to as routine medium. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 until 2 weeks post-confluency. Twenty four hours before the start of an experiment, the remaining explant was removed and culture medium was replaced with routine medium devoid of insulin and FCS, but supplemented with 0.1% bovine serum albumin, 4 µM spermine and spermidine, and 1 µM ethanolamine (referred to as serum-free medium [SF]). Experiments were performed in serum-free medium.

Confluent culture model mimicking intact urothelium

After reaching confluency, cultures were maintained in routine medium for an additional two weeks to ensure proper differentiation of the whole culture. Before treatment with growth factors, cultures were rinsed twice with SF followed by incubation in SF for 24 hours.

Subsequently, medium was replaced with SF with or without growth factor for another 96 hours. Medium was replaced daily during the course of the experiments.

Culture model mimicking reepithelializing urothelium

Injuries were made directly after the 24-hours incubation with SF. Four circular imprints were made with a biopsy punch (Stiefel, Offenbach am Main, Germany; Ø 4mm) [6]. The urothelium within the injured areas was scraped manually under a dissecting microscope . After injury, the cultures were washed twice with SF followed by incubation of the cultures with SF with or without the addition of growth factors. At different time-points during cultivation, the wound edge was monitored through a light microscope. From the acquired image, the uncovered area was measured and expressed as percentage of the initial wound area.

In the experiments, where the *de novo* c-erbB2-protein production was blocked using an antisense oligonucleotide strategy, 24 hours before damage, routine medium was replaced with SF containing 5 nmol/ml oligonucleotides. After 24 hours, damages were made as described. During the next 48 hours, medium with our without oligonucleotides was replaced twice. Phosphorothioated oligonucleotides were obtained in collaboration with Dr. M. Lemâitre (Eurogentec, Liège, Belgium) based on experiments described by Vaughn *et al.* [35]. Sequences of the respective C-erbB2 oligonucleotides are: antisense oligonucleotide: 5'-GAGGTACCACGAGTG-3'; mutated variant of the antisense oligonucleotide: 5'-GAGTGACCACAGGTG-3'; sense oligonucleotide: 5'-CTCCATGGTGCTCAC-3'.

Proliferation assay

Two hours before termination, cultures were incubated with 40 µg/ml bromodeoxyuridine (BrdU). Subsequently, cultures were rinsed with PBS, pH 7.2, and fixed with 70% ethanol followed by immunohistochemistry. Eight prefixed areas of 0.15mm^2 per culture were counted. Four areas just outside each wound and another four areas within the wound that were covered by regenerative urothelium were counted. The BrdU-incorporation was expressed as the labeling index: the relative number of BrdU-positive nuclei in four prefixed areas of 0.15mm^2 per culture.

Immunohistochemistry

The immunostaining of the cultures was performed as described [16]. The expression was visualized using appropriate dilutions of the primary antibodies in a conjugated immunoenzyme assay. Secondary antibodies were either peroxidase or alkaline phosphatase conjugated. As substrates, we used 3,3'-diaminobenzidine tetrahydrochloride or naphtol AS-MX phosphate. The level of c-erbB-receptor expression was determined semi-quantitatively, with 0 = no expression; 1 = low expression; 2 = moderate expression; 3 = high expression; 4 = very high expression [14].

Statistics

Experiments were performed at least in triplicate. A student's t-test was used to determine the statistical significance of the data. At $p<0.05$, data were accepted as statistically significant. Data are represented as mean ± standard error.

Results

Confluent cultures representing intact urothelium

As shown in Figure 1A, treatment with amphiregulin and TGFα, significantly enhanced the proliferation of the urothelial cultures three to four times as visualized by the labeling index ($p<0.003$). In contrast, treatment with HRGα or EGF did not stimulate urothelial proliferation. The cellularity of the cultures is increased upon treatment with amphiregulin, TGFα or EGF (Fig. 1B). The fact that EGF induces an enhanced cellularity of the cultures must imply that EGF had transiently stimulated proliferation before the addition of BrdU or that EGF could inhibit apoptosis in these urothelial cultures, as has previously been described for other cell cultures [11, 21, 34].

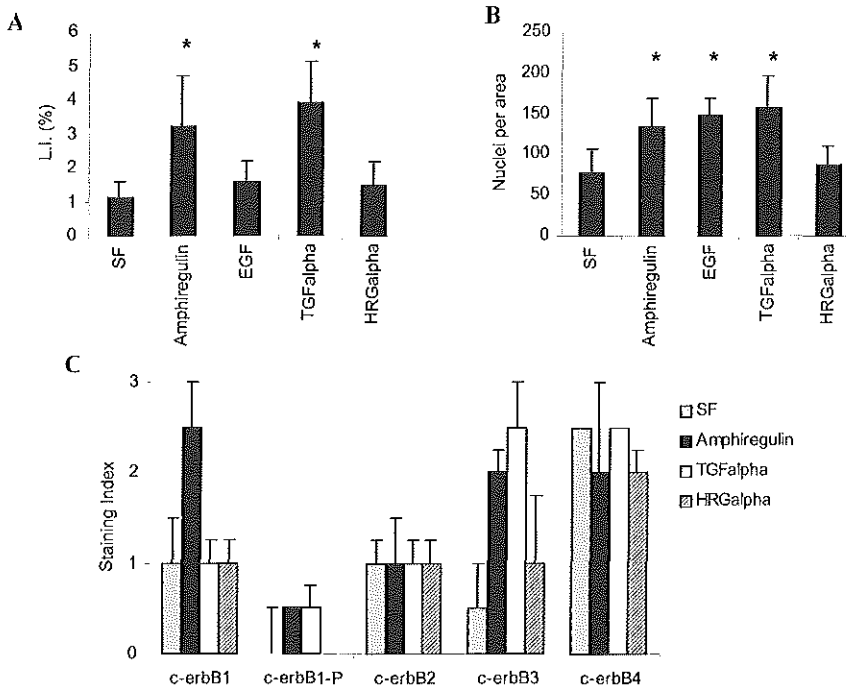

Figure 1: Effect of EGF-like growth factors on (A) the labeling index, (B) the number of nuclei and (C) the expression of erbB-family members in confluent primary urothelial cultures. An asterisk indicates a significant difference ($p<0.05$) as compared with SF-medium. C-erbB1-P = antibody against phosphorylated C-erbB1.

The amphiregulin and TGFα mediated induction of proliferation is associated with an enhanced expression of c-erbB3. The constitutive expression of c-erbB2 and c-erbB4 was not changed upon growth factor treatment. The constitutive expression of c-erbB1 was only induced by amphiregulin, whereas the expression of phosphorylated c-erbB1 was slightly enhanced by both amphiregulin and TGFα (Fig. 1C).

Given the upregulation of both phosphorylated c-erbB1 as well as c-erbB3 by amphiregulin and TGFα, it is tempting to hypothesize that amphiregulin, EGF, or TGFα mediate their effects also via c-erbB3. Furthermore, the expression of c-erbB4 in the intact cultures is constitutively high irrespective of the growth factor treatment, and it is known that c-erbB3 has a high affinity for c-erbB4 to form a heterodimer [8]. However, HRGα, which primarily signals through the c-erbB3/c-erbB4 heterodimer, has no proliferative effect on human urothelial cells (Fig. 1A).

Regenerating urothelial cultures

Under serum-free conditions, approximately 84% of the wounds is covered with new urothelium within 48 hours. The reepithelialized area is markedly enlarged upon treatment with TGFα or EGF, and to a lesser extent, by amphiregulin (Fig. 2). In fact, when treated with EGF or TGFα, wounds completely closed within 48 hours. Treatment with HRGα has no effect on urothelial regeneration as compared to nontreated cultures.

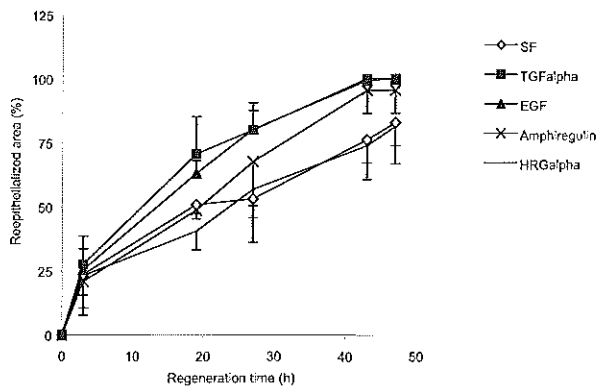

Figure 2: Influence of EGF-like growth factors on reepithelialization of injured urothelial cultures.

In general, growth factor treatment has no stimulating effect on urothelial proliferation during the first 24 hours of wound healing (Figs. 3A and 3B). During the next 24 hours, a

In general, growth factor treatment has no stimulating effect on urothelial proliferation during the first 24 hours of wound healing (Figs. 3A and 3B). During the next 24 hours, a gradual increase in proliferation was noted. Nevertheless, labeling indices remained relatively low. Therefore, the observed stimulation of reepithelialization after EGF, TGFα or amphiregulin treatment could not be explained by a significant induction of proliferation. This suggests that the induced reepithelialization could only be explained by an enhanced migration of cells into the denuded area.

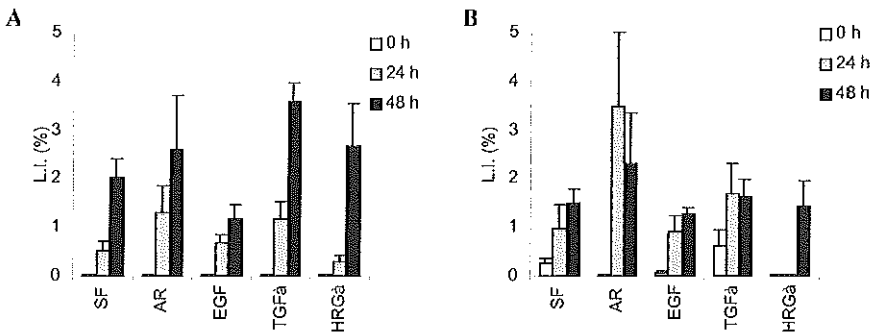

Figure 3: Effect of EGF-like growth factors on the proliferation of injured urothelial cultures. BrdU incorporation was determined in prefixed regions (A) just outside the injury and (B) within the reepithelialized wound-area.

The stimulated wound closure may be accompanied by an enhanced expression of specific growth factor receptors. According to other functional studies, EGF-like factors mediate their effects mainly via c-erbB1, c-erbB2, and c-erbB4. Therefore, we quantitated the immunohistochemical expression of c-erbB1, c-erbB2 and c-erbB4. The c-erbB1 expression is low and is not significantly affected by any of the investigated growth factors during any point of the repair-process (data not shown). The c-erbB2 expression tends to be higher only in cells near the edge of the damaged area upon treatment with amphiregulin, TGFα or EGF (Figs. 4A and 4B). The c-erbB4 expression shows no significant differences during wound healing (data not shown).

Our data points to a function of not only c-erbB1, but also of c-erbB2 in the epithelial regeneration. To investigate whether c-erbB2 is necessary for the reepithelialization, we treated cultures with antisense oligonucleotides for c-erbB2 and examined the regeneration during this treatment.

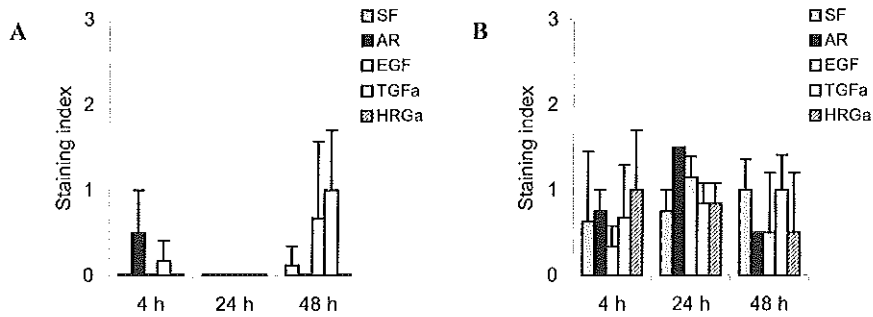

Figure 4: Expression of c-erbB2 in EGF-like growth factor treated injured urothelial cultures. C-erB2-expression was determined at various time-points in regions (A) just outside the injury and (B) within the reepithelialized wound area.

Treatment of injured cultures with c-erbB2 antisense DNA

Treatment of damaged cultures with c-erbB2 antisense oligonucleotides significantly inhibited the reepithelialization up to 50% (Figs. 5 and 6A). The sense and the mismatched variant of the antisense oligonucleotides did not affect the regeneration (Figs. 5, 6B and 6C).

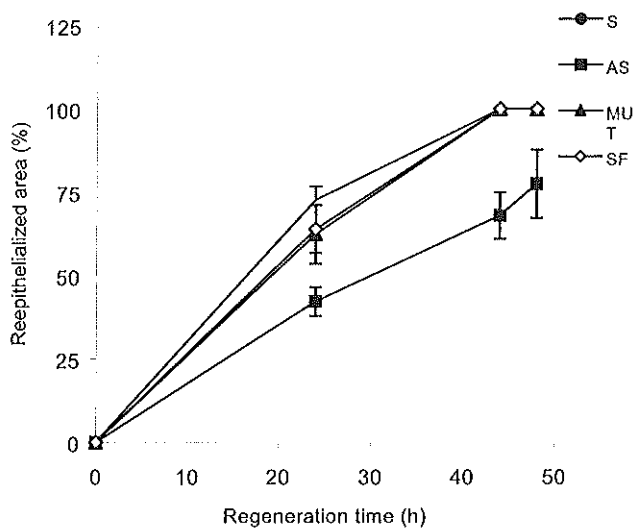

Figure 5: Influence of c-erbB2 oligonucleotides on the reepithelialization of injured urothelial cultures.

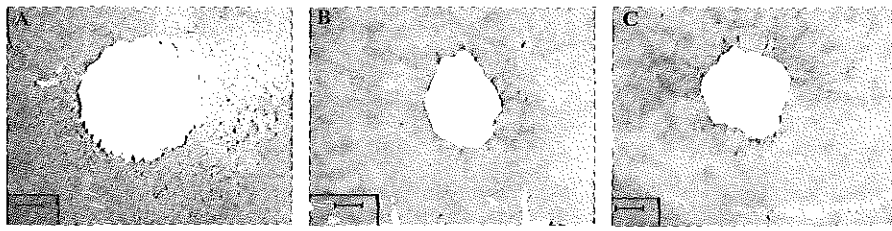

Figure 6: Topview of standardized injuries made in primary cultures of human urothelium. Effect on reepithelialization after treatment for 24 hours with c-erbB2 oligonucleotides: (A) antisense DNA, (B) sense DNA and (C) mutated variant of antisense DNA.

This indicates that the wound healing was specifically perturbed by the c-erbB2 antisense only. The proliferation was similar in all damaged cultures (Figs. 7A and 7B) suggesting that this delay in reepithelialization could not be explained by a diminished proliferative capacity of the antisense treated cultures. Also, none of the control oligonucleotides had a significant effect on proliferation.

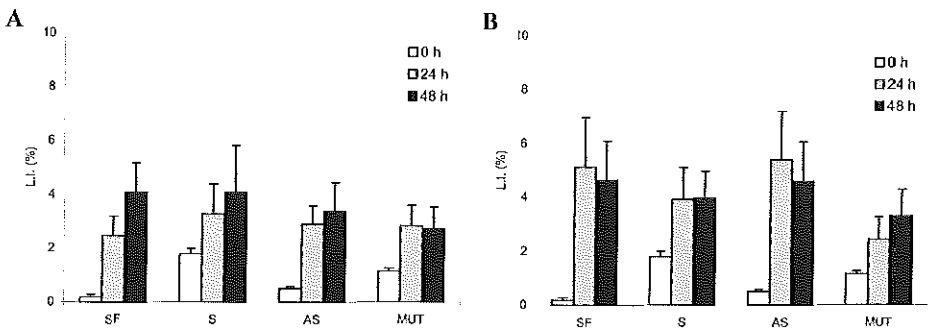

Figure 7: Effects of c-erbB2 oligonucleotides on the BrdU incorporation, determined (A) just outside the injuries and (B) within the reepithelialized area.

Discussion

Earlier studies on the functional effects of EGF-like growth factors on urothelium are conflicting. Previously, we have shown that the growth and regeneration of murine urothelium *in vitro* was stimulated by TGFα or EGF [6, 15, 31]. Furthermore, *in vivo* studies indicated that EGF induced urothelial proliferation and hyperplasia in rats and pigs [26, 37]. However, others have claimed that despite the presence of EGF-receptors on human urothelium [16, 27], EGF was not essential for the growth of normal human urothelial cells *in vitro* [9, 20, 27, 33].

In this study, using a previously described *in vitro* organotypic model for human urothelium, amphiregulin, TGFα and EGF stimulated the growth of intact human urothelium. No functional effects of amphiregulin on the growth of human urothelium have been reported previously. But Cilento *et al.* showed that human urothelial cells in culture can produce high levels of amphiregulin mRNA, suggesting that amphiregulin is involved in the autocrine growth regulation of urothelium [9].

We demonstrated that urothelial reepithelialization is enhanced by the EGF-like factors TGFα, EGF and amphiregulin but not by HRGα. The mode of action of TGFα/EGF/ amphiregulin may be mainly through interaction with EGFR which either transduces the signal by homodimerization or by heterodimerization with c-erbB2. Treatment of the regenerating urothelial cultures with c-erbB2 antisense DNA confirmed that c-erbB2 might be involved in urothelial regeneration. The role of c-erbB3 during the EGF/TGFα/amphiregulin stimulated reepithelialization is not yet clear as these factors do not bind to c-erbB3. Though, heterodimerization of c-erbB1 or c-erbB2 with c-erbB3 may attribute to the observed effect [1, 28]. An alternative role for c-erbB3 is that endogenously expressed HRGα may stimulate the proliferation in a c-erbB3-dependent way. Furthermore, the constitutive high expression of c-erbB4 during urothelial reepithelialization is suggestive for a role of c-erbB4 in urothelial maintenance and/or regeneration. Future functional *in vitro* studies may clarify possible functions of these respective EGFR family members in urothelial regeneration.

In the same model of urothelial regeneration, Daher *et al.* recently showed that reepithelialization could also be inhibited by a functional blocking EGFR antibody or by the EGFR-tyrosine kinase inhibitor Tyrphostin AG1478 [10]. In their study, these EGFR inhibitors affected both proliferation and migration in urothelial wound repair. Our combined data indicate that in damaged urothelium both EGFR and c-erbB2 are involved in the process of reepithelialization.

Our experiments with the antisense c-erbB2 oligonucleotides suggest that c-erbB2 is an important mediator of migration during wound healing. Few studies reported on the role of the c-erbB2 protein in cell migration. Overexpression of c-erbB2 in breast or ovarian carcinoma cells or fibroblasts resulted in an increased cell migration [18, 36] or invasion [13]. Furthermore, cytoplasmatic expression of p185-neu in human astrocytoma cells is associated with a high degree of migratory activity [5]. The exact mechanisms of c-erbB2-enhanced migration are only poorly understood. A direct mechanism could be via signaling through the Ras-MAP kinase pathway [3] ultimately leading to transcription of genes involved in cell migration. Indirectly, it may be caused by downregulation of E-cadherin or α2-integrin [19] or by activation of the PEA3 transcription factor that in turn activates genes, which encode enzymes required for cell migration [4].

In conclusion, the EGF-EGFR pathway is involved in the normal growth and regeneration of human urothelium. Amphiregulin, EGF and TGFα might be involved in the (autocrine) growth regulation of urothelium. Enhanced expression of some of these growth factors and their receptors could contribute to the deregulated growth of bladder tumors, making them suitable as targets for future developments in cancer therapy. On the other hand, EGF and TGFα are important mediators of reepithelialization, suggesting that concise use of EGF-family members could aid regeneration of damaged urothelium, and improve bladder reconstruction and replacement surgery.

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Chapter 4

E-CADHERIN PROMOTES INTRAEPITHELIAL EXPANSION OF BLADDER CARCINOMA CELLS IN AN *IN VITRO* MODEL OF CARCINOMA *IN SITU*

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Abstract

High-grade transitional cell carcinomas (TCC) of the urinary bladder are frequently associated with carcinoma *in situ*, which may replace large areas of the mucosa of the urinary tract. The invasive component of TCC often reveals a loss of expression of the cell-cell adhesion molecule E-cadherin, but the role of E-cadherin in the development and expansion of intraepithelial neoplasia is unknown. To study the underlying mechanism of intraepithelial expansion we have developed an intraepithelial expansion (IEE) assay. Human TCC cell lines were investigated in this IEE assay for their capacity to replace the surrounding normal murine urothelial cells. *In vitro* IEE appeared to be prominent in three (SD, RT112, and 1207) of the four E-cadherin positive cell lines. Although the two E-cadherin negative cell lines (T24 and J82) were able to penetrate surrounding normal urothelium as single cells, they largely lacked the capacity of IEE. These results prompted us to investigate whether the cell-cell adhesion molecule E-cadherin is an important determinant for IEE. T24 cells transfected with full-length mouse E-cadherin cDNA, displayed an enhanced intraepithelial expansion rate. Transfection did not influence their proliferative capacity, or their pattern and level of integrin expression, or their ability to expand in the absence of surrounding urothelium. The data suggest that E-cadherin mediated cohesiveness is an important factor in the intraepithelial expansion of bladder carcinoma cells. These observations argue for a dual, paradoxical role of E-cadherin in bladder tumorigenesis. On the one hand, E-cadherin promotes the expansion of intraepithelial neoplasia; on the other hand, its loss correlates with invasive behavior.

Introduction

High-grade transitional cell carcinomas (TCC) of the urinary tract are frequently accompanied by carcinoma *in situ* (1). Carcinoma *in situ* of the urinary tract is characterized by the replacement of the normal lining urothelium by dysplastic cells, which show a variety of cellular and molecular changes. Carcinoma *in situ* may replace large areas of the urinary tract mucosa, extending even to the urethra and – in males - to the prostatic ducts and glands (2). In general, carcinoma *in situ* is regarded as a precursor lesion for invasive bladder carcinoma (3).

Patients with carcinoma *in situ* in the flat peripheral urothelium adjacent to tumors have a higher probability of tumor recurrences and/or invasion (4,5). Clinical and experimental data suggest that bladder tumor recurrences could be the consequence of intraepithelial expansion of the transformed cells from the original tumor or shedding and subsequent reattachment of bladder tumor cells particularly to traumatized areas in the bladder mucosa (6-11). Lateral expansion of the attached tumor cells can then lead to the replacement of normal urothelium by cancer cells. The mechanisms involved in lateral (i.e. intraepithelial) expansion of bladder tumor cells are only partially understood. A cocultivation model established recently in our laboratory permits the direct visualization of attachment and subsequent intraepithelial expansion (IEE) of bladder tumor cells at the expense of surrounding normal urothelium (10,11). In this assay, a tumor cell suspension is inoculated on confluent mouse urothelial cultures containing de-epithelialized areas of a standard size. Implantation of tumor cells occurs predominantly in these de-epithelialized areas and the time course of IEE can be assessed by selective immunostaining of the tumor cells. Previously, we have shown in this model that exposure to growth factors and culture on substrates coated with particular extracellular matrix proteins could influence IEE (10,11).

E-cadherin is a member of a family of transmembrane glycoproteins involved in intercellular adhesion. E-cadherin function is mediated by the interaction with the cytoplasmatic α-, β- and γ-catenins. These catenins connect E-cadherin with the cytoskeleton. In model systems, loss of E-cadherin expression is associated with the gain of the invasive phenotype in tumors (12-14). Similarly, it was reported that loss of the invasion suppressor molecule E-cadherin or catenins is associated with deeply invasive bladder cancer and is predictive for poor survival of patients with bladder cancer (15-17). The E-cadherin/catenin complex also contributes to a variety of physiological functions like cell growth,

differentiation, wound healing, cell motility, morphogenesis, and organogenesis (18). However, its role in the expansion of carcinoma *in situ* of the bladder has not yet been studied.

In this study, initial experiments comparing E-cadherin-positive and -negative human TCC cell lines suggested a potentially enhancing role of E-cadherin in IEE. By use of T24 cells, stably transfected with an E-cadherin cDNA construct, we could confirm that this molecule indeed contributes to IEE most likely by conveying increased cohesiveness to the TCC cells.

Material and methods

Cell culture

The human bladder cancer cell lines T24, SD, RT112, JON, and J82 were kindly provided by Prof. Dr. J.A. Schalken (Urological Research Laboratory, University Hospital Nijmegen, The Netherlands) and have been characterized previously (19,20). Human bladder carcinoma cell line 1207 was obtained from Dr. W.I. De Boer (GETU Service d'Urologie, Paris, France; Ref. 21). Cell lines were maintained in the same standard medium as the primary bladder explant cultures.

Primary explant cultures of murine urothelium

Murine urinary bladders were dissected from female C3H/He mice, 6-8 weeks of age, and cut into halves. The mucosa of the bladder was stripped from its underlying muscle layer and subsequently spread on a collagen type IV (25 µg/ml human collagen type IV)-coated Cyclopore membrane (Becton Dickinson Labware, Bedford, USA) with the submucosa facing the culture support. Standard culture medium consisted of a 1:1 mixture of DMEM and Ham's F10, supplemented with 10% heat-inactivated FCS, 10 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenite, 10 µM HEPES, 50 nM hydrocortisone, 100 IU/ml penicillin and 100 µg/ml streptomycin. The explant cultures were grown at 37° C in a humidified atmosphere of 5% CO_2. These murine explant cultures on porous membranes mimic the *in vivo* situation; the cultured urothelium shows a polarized multilayering and differentiation into umbrella cells (22).

Intraepithelial expansion (IEE) assay

The cocultivation model to study IEE of bladder carcinoma cells was described previously (11). Briefly, in confluent murine explant cultures four standardized circular areas were denuded in the periphery of the primary cultures by cautious imprinting with a 3-mm diameter biopsy punch (Stiefel, Offenbach am Main, Germany). The urothelium in the injured areas was scraped away from the cyclopore membrane with a micropipette tip. Subsequently, the cultures were washed twice with PBS, followed by seeding of 10^5 tumor cells in 1.5-ml standard medium on the murine urothelial explant cultures. The bladder tumor cells were allowed to attach to the injured areas in the explant cultures for 24 hours; nonadherent cells were then washed away with PBS, and the cultures were either terminated or continued for

another 4, 7, or 14 days in standard medium. Each experiment was performed twice in triplicate. The cultures were terminated by fixing them in 70% ethanol and stored at 4°C until immunohistochemistry for selective identification of the human bladder carcinoma cells was performed.

Monoclonal, human-specific antibodies, RCK108 (Eurodiagnostica, Arnhem, The Netherlands) or DC10 (Beckman Coulter, Fullerton, CA, USA), directed against cytokeratin 19 were used to distinguish the human bladder carcinoma cells from murine urothelium (10). Monoclonal antibody DC10 was used for staining J82 cells, whereas the other cell lines were stained with monoclonal antibody RCK108. Nearly 100% of the bladder carcinoma cells of each cell line was labeled with RCK108 or DC10. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Dako, Glostrup, Denmark). Peroxidase activity was visualized with 0,03% H_2O_2 and 0,02% 3,3,-diaminobenzidine tetrahydrochloride (Fluka, Basel, Switzerland) diluted in PBS.

The immunostained areas were quantitated with a Hitachi CCTV camera equipped with the KS400 image analysis software package (Kontron Elektronik, Eching, Germany), and expressed in mm^2.

Construction of stable transfectants

The E-cadherin-negative cell line T24 was cotransfected with plasmids pBATEM2 and pSVneo, which harbor the neomycin resistance gene. Marion Bussemakers (Urological Research Laboratory, University Hospital Nijmegen) generously provided plasmid pBATEM2, containing the full-length mouse E-cadherin cDNA, originally constructed by Nagafuchi *et al.*(23). For transfection, cultures were cotransfected with a total of 20μg of DNA (pBATEM2: pSVneo, 20:1) using the DNA-calcium phosphate method (24), and transfected cells were selected with 800 μg/ml G418. E-cadherin expression in these transfected cell lines was detected as follows. Clones were grown to confluence on multichamber slides (Nunc, Naperville, USA) and fixed in methanol at -20°C. Cells were incubated overnight at 4°C with monoclonal antibody DECMA-1 (Sigma, St. Louis, USA), followed by a biotinylated rabbit anti-rat immunoglobulins (Dako) and fluorescein-conjugated streptavidin (Dako). The different clones retained their resistance to G418 and were stable in their E-cadherin expression during the entire course of the described experiments.

Flow cytometric analysis of E-cadherin and integrin expression

Cells were harvested by a short trypsinization of confluent monolayers. Cell suspensions were made in PBS containing 0.5% BSA, 0.1% NaN_3, 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at a concentration of 1 x 10^6 cells/ml. E-cadherin expression of a selection of nine different, transfected T24 clones was examined with the DECMA-1 antibody, followed by biotinylated rabbit anti-rat immunoglobulins and fluorescein-conjugated streptavidin. Integrin expression was studied with the use of specific antibodies against α1-integrin (HP2B6, Beckman Coulter, Fullerton, CA, USA) α2-integrin (NCL-CDW49b, Novacastra, Newcastle upon Tyne, UK), α3-integrin (NCL-CDW49c, Novacastra), α4-integrin (P4G9, Dako), α5-integrin (P1D6, Dako) α6-integrin (NCL-CDW49f, Novacastra), β1-integrin (TDM29, Sanbio, Uden, The Netherlands) and β3-integrin (NCL-CD61, Novacastra). As a negative control, the primary antibody was omitted and replaced by a PBS/BSA/Azide solution. Data acquisition and analysis were performed on duplicate samples on a FACScan flow cytometer using CELLQuest software (Becton Dickinson, San Jose, CA, USA).

Immunoblotting

Confluent cultures were lysed in 2X sampling buffer [4% SDS, 200 mM DTT, 100mM Tris (pH 6.8), 20% glycerol, and 2% Triton X-100], and equal quantities of protein (30μg) were run on a 7.5% SDS-PAGE. The MDCK cell line was used as a positive control for E-cadherin expression. After electroblotting, blots were immunostained with the DECMA-1 antibody, followed by biotinylated rabbit anti-rat immunoglobulins. Next, an alkaline phosphatase-conjugated streptavidin label (Biogenex, San Ramon, USA) was applied. Finally, bound antibody was visualized by histochemical staining with NBT/BCIP ([nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt] Roche Diagnostics, Basel, Switzerland).

In vitro invasion assay

Chicken heart invasion assays were performed as described by Marcel *et al.* (25). Briefly, a selection of E-cadherin transfected T24 clones was confronted with precultured rounded fragments of embryonic chicken heart on soft agar for 24 hours. Next, fragments with attached bladder tumor cells were kept in suspension culture under gyrotory shaking (120 rpm; 37°C; 5% CO_2) for 6 days in MEM REGA 3 medium (GibcoBRL/ Life Technologies, Breda, The Netherlands) containing 10% FCS. Fragments were fixed in 4% phosphate

buffered formalin and embedded in paraffin. Invasion was scored on serial histological sections stained with hematoxylin/eosin. The tumor cells were distinguished from the heart tissue by their morphological features.

Wound colonization assay

T24 Clones were grown to confluence in 10-cm ∅ culture dishes. With a plastic pipette tip, cells were scraped away in the shape of a cross. The width of the lesion was approximately 5mm. After 16 hours the movement of cells into the wound was monitored and photographed.

Expansion of T24 clones on collagen type IV-coated cyclopore membranes

With a 3-mm ∅ biopsy punch (Stiefel, Offenbach am Main, Germany), a superficial circular imprint was made on the cell culture inserts, coated with collagen type IV. A cell suspension of 10^5 cells/2µl standard medium was pipetted within the borders of the circular imprint with a 10-µl micropipette. The cells in the drop of medium attached into the circular area within 24 hours. Every two days medium on top of the membranes was refreshed, taking care that the medium only covered the area of tumor cells. Medium underneath the membrane was refreshed twice a week. The circumferences of the outgrowing sheet of tumor cells were drawn daily. From these drawings, the area (mm^2) of outgrowth was determined.

[^{3}H]Thymidin incorporation

To assess the potential differences in proliferation of the different T24 clones, a cell kinetic study was performed as described earlier (11). Briefly, T24 clones grown in 75 cm^2 culture flasks were synchronized in serum-free culture medium for 24 hours. Subsequently, 10^5 cells/well were seeded in collagen type IV-coated 96-wells dishes and were cultured in standard medium for 4 consecutive days. Proliferative activity was determined at 24, 48, 72, and 96 hours. During the final 16 hours of culture, cells were incubated with 0.5 µCi [^{3}H]thymidin/well. The incorporated [^{3}H]thymidin in harvested cells was counted in a BetaPlate scintillation counter (LKB-Pharmacia, Woerden, The Netherlands).

Results

IEE of human bladder carcinoma cells

The IEE of a panel of six different TCC cell lines was studied. After the seeding of tumor cells on wounded confluent murine urothelial cultures, the tumor cells preferentially attached to the de-epithelialized areas. Within 24 hours, these areas were covered both by tumor cells and regenerating urothelium. Control experiments showed that in the absence of inoculated tumor cells, the de-epithelialized areas became entirely covered by regenerating normal urothelial cells within 24 hours (11). The areas covered by tumor cells at 24 hours were of the same size for four of the six examined tumor cell lines, but implantation of JON or J82 cells was less effective. As described previously, E-cadherin expression influenced the pattern of IEE (10,11). Bladder tumor cell lines with no (T24 and J82) or a heterogeneous (1207) expression of E-cadherin were able to infiltrate the surrounding normal urothelium as single cells (Fig. 1A), whereas bladder tumor cell lines with a homogeneous (SD, RT112, and JON) expression of E-cadherin displayed a sharp demarcation between the tumor cells and the normal urothelium (Fig. 1B).

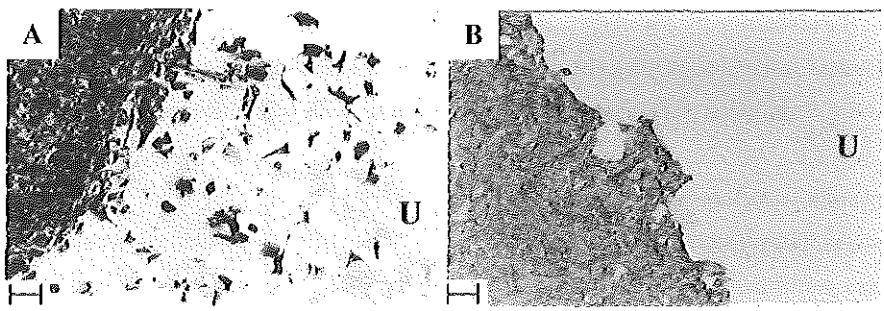

Figure 1:Low power overviews of human bladder carcinoma cells adjacent to primary normal mouse urothelium: A) T24 cells and B) RT112 cells. Bladder carcinoma cells were selectively stained with RCK108. Scale bar = 100μm. (U=unstained normal surrounding mouse urothelium.).

The 6 tumor cell lines further differed with respect to their subsequent IEE rate (Fig. 2 and Table 1). The IEE of bladder carcinoma cells was most pronounced for the SD cells and less for the 1207 and RT112 cells, whereas T24, JON, or J82 displayed hardly any or no expansion. Therefore, three of four E-cadherin-positive bladder carcinoma cell lines had a

better IEE rate than the two E-cadherin-negative bladder carcinoma cell lines. These results suggested a positive effect of E-cadherin on IEE.

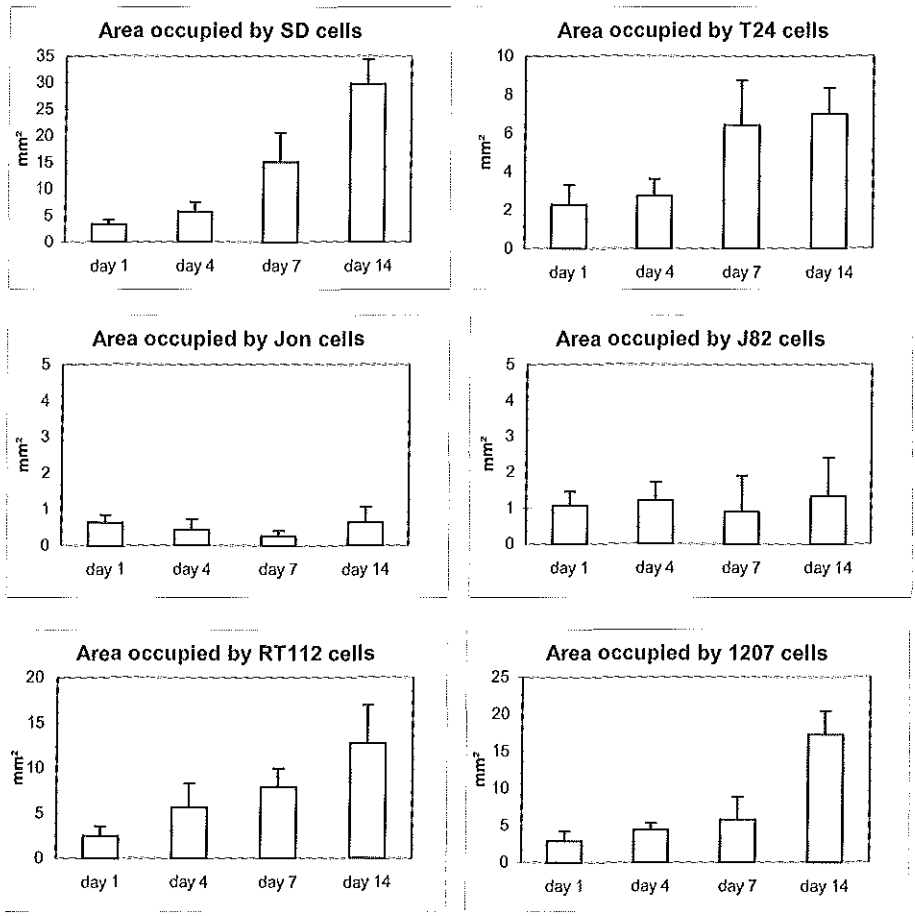

Figure 2: IEE of 6 different bladder tumor cell lines.
Graphs display the area (mm²) occupied by human bladder tumor cells in peripheral lesions measured on different time points. Data are expressed as means ± SD (n=6 cultures, with four peripheral lesions each). Note the difference in the scale of the y-axis in the various graphs.

Table 1 Characteristics of IEE of bladder carcinoma cell lines

Cell line	E-cadherin expression (*in vitro*)	IEE	
		Tumor expansion	Infiltration pattern
SD	+	++	Sharp demarcation
T24	–	–/+	Single cells
RT112	+	+	Sharp demarcation
J82	–	–	Single cells
JON	+	–/+	Sharp demarcation
1207	–/+	+	Single cells

Transfection of T24 with full-length mouse E-cadherin cDNA

To investigate whether E-cadherin is involved in the IEE of bladder tumors, we transfected T24 cells with the mouse E-cadherin gene. Earlier, it was shown that T24 cells express α- and β-catenin (25). We obtained nineteen G418-resistant T24 cell clones, with membrane-bound E-cadherin expression (Fig. 3A).

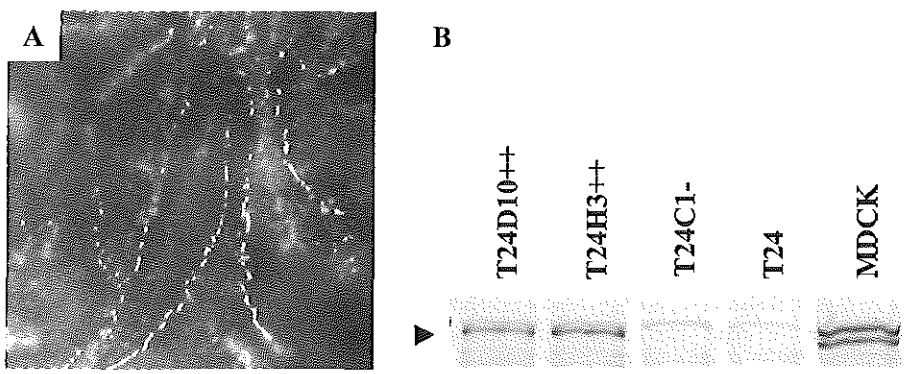

Figure 3: Analysis of E-cadherin expression in transfected T24 clones. Immunofluorescent staining for E-cadherin in T24D10++ (A) [magnification 100X]. Western blotting of total lysates of MDCK, T24, and T24 transfectants (B).

Clones varied with regard to their levels of immunohistochemically detected E-cadherin expression. Expression levels of E-cadherin of nine different T24 transfectants were analyzed by flow cytometry. Clones T24H9+, T24 2D4+, T24D4+ and T24H5+ had low to moderate expression levels (Fig. 4B), whereas the clones T24H3++ and T24D10++ had a high expression level of E-cadherin (Fig. 4C). As a negative control for E-cadherin expression, we used the clones (T24C1-, T24F1-, and T24G1-) that were G418 resistant but had no detectable expression of E-cadherin either by immunofluorescence, immunoblotting, or flow cytometry (Fig. 4A). Immunoblotting with the DECMA-1 antibody on different cell lysates of T24 clones showed a characteristic band at 120 kD that was typical of the E-cadherin-positive clones (Fig 3B). At the ultrastructural level no difference in the presence of desmosomes between the nontransfected (E-cadherin-negative) and transfected, E-cadherin-positive T24 cells was noted (data not shown).

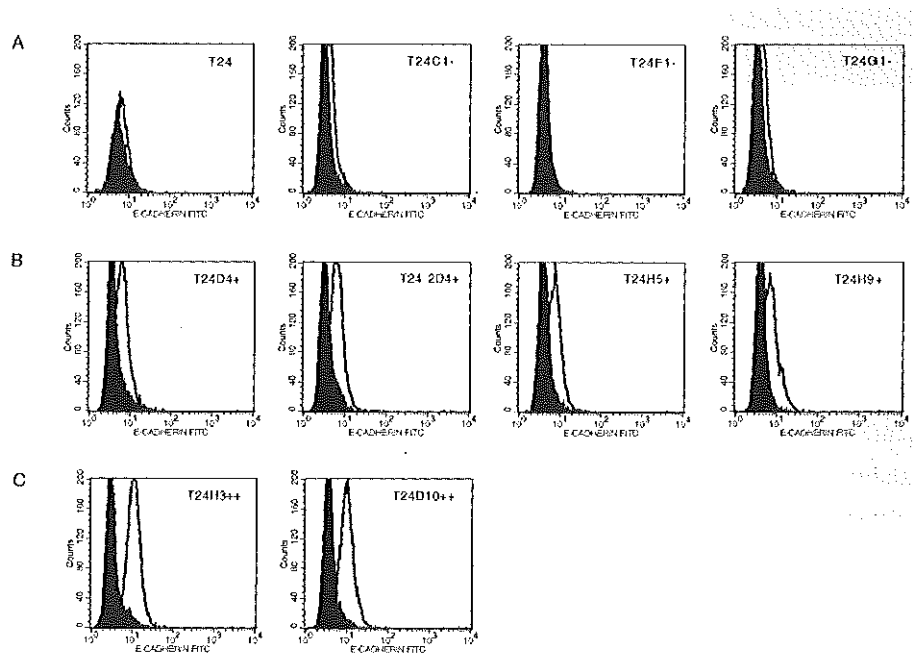

Figure 4: Flow cytometry analysis of E-cadherin expression in A) native T24 cells and the negative controls T24C1-, T24F1-, T24G1-; B) Clones T24D4+, T24 2D4+, T24H5+, T24H9+ and C) clones T24D10++ and T24H3++.

Wound colonization assay

The capacity to repair lesions in a confluent cell culture was monitored for the different clones. Strikingly, untransfected T24 cells and the T24 clones T24C1-, T24F1- and T24G1-, with no E-cadherin expression, migrated into the denuded area as single cells (Fig. 5A). SD and the T24 clones with a high or moderate E-cadherin expression filled up the lesions by moving as a cohesive sheet (Fig. 5B and Table 2). However, no difference in time required for the repair of the lesions was seen.

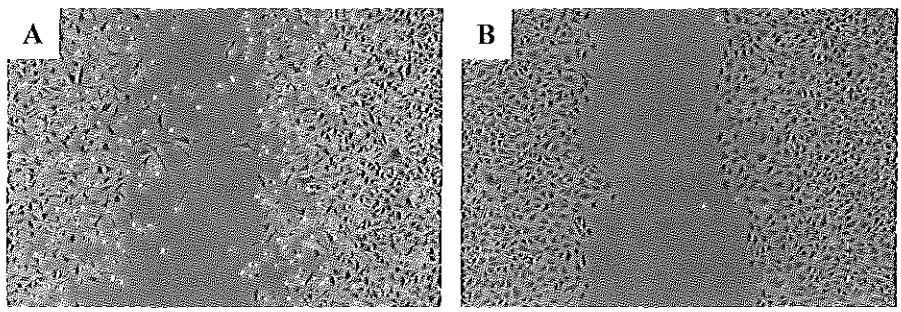

Figure 5: Wound colonization of transfected T24 cells. The photographs illustrate the difference in wound-filling capacity between the E-cadherin-negative T24 clone, T24G1- (A) and an E-cadherin-positive clone, T24D10++ (B).

In vitro embryonic chicken heart invasion assay

In vitro invasion of bladder carcinoma cells was examined after 6 days of cocultivation with embryonic chicken heart fragments. As a negative control, we used the E-cadherin-positive SD cell line. Tumor cell adhesion to chicken heart fragments varied from 100% for T24D10++ and T24H3++ cells to 65% for the T24 cells (Table 2). Untransfected T24 cells exhibited the strongest invasive capacity. After successful adhesion, T24 tumor cells infiltrated nearly all embryonic chicken heart fragments (87%). SD cells were unable to invade the embryonic chicken heart fragments at all. Transfection of E-cadherin in T24 cells resulted in a significant reduction of invasion for T24D10++ and T24H3++ (Table 2). Both cell lines had a high expression of E-cadherin. Clones T24H9+, with a moderate expression of E-cadherin, T24C1- and T24G1-, with absent E-cadherin expression, exhibited a moderate to high invasive capacity into the embryonic chicken heart fragments. These results revealed the levels of E-cadherin expression of the transfected T24 clones corresponded with their functionality.

Table 2 *In vitro* invasion of E-cadherin-transfected T24 cells

Cell line	E-cadherin expression of cultured cells	*In vitro* confronting cultures		Wound colonization
		Adherent[a]	Invasive[b]	
SD	++	20/23 (87%)	0/20 (0%)	Cohesive sheet
T24	-	15/23 (65%)	13/15 (87%)	Single cells
T24D10++	++	34/34 (100%)	3/34 (9%)	Cohesive sheet
T24H3++	++	11/11 (100%)	2/11 (18%)	Cohesive sheet
T24H9+	+	21/22 (95%)	7/21 (33%)	Cohesive sheet
T24G1-	-	21/21 (100%)	11/21 (52%)	Single cells
T24C1-	-	20/21 (95%)	15/20 (75%)	Single cells
T24H5+	+	11/12 (92%)	3/11 (27%)	Cohesive sheet

a) Number of adherent / total number of confronted embryonic chicken heart cultures;
b) Number of invasive / number of confronted cultures with adherent tumor cells.

IEE of transfected T24 cells

The SD cell line was used as a positive control, because it had the highest expansion rate of the TCC cell lines (Fig. 2). Transfection of T24 cells with mouse E-cadherin cDNA stimulated IEE of two independently obtained clones, T24D10++ and T24H3++, both with a high expression of the E-cadherin protein (Fig. 6).

Clones with a moderate/low expression level of E-cadherin (*e.g.*, T24H9+) did not show a significant stimulation of IEE. The implantation (defined by the tumor area on day 1) of the different T24 transfectants was comparable, indicating that possible differences in IEE could not be attributed to differences in attachment of the T24 transfectants to the wounded area.

The expression of integrins was determined by flow cytometry in T24, T24H3++, T24D10++ and T24C1-. In the four tested clones, no differences were observed in expression levels of α1-, α2-, α3-, α4-, α5-, α6-, β1-, or β3-integrins (data not shown).

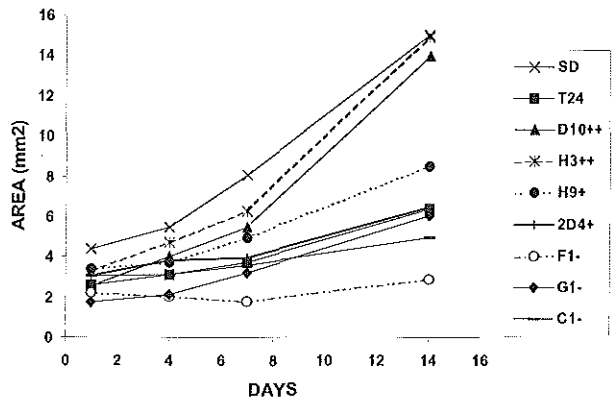

Figure 6: IEE of T24 transfectants. Graphs display the area (mm²) occupied by human bladder tumor cells in peripheral lesions measured on different time points. Data are expressed as means (n=4/5 cultures, with four peripheral lesions each).

Expansion of T24 clones on collagen type IV-coated cyclopore membranes in the absence of surrounding urothelium

We tested whether the observed differences in IEE rate between the tested T24 clones could be explained by an altered expansion rate (expansion defined as the outgrowth of a sheet of cells) or proliferative capacity. The expansion of different T24 clones in the absence of surrounding urothelium was assessed (Fig. 7). These experiments revealed no differences in the outgrowth or in the [^{3}H]thymidine incorporation (data not shown) of the various T24 clones. The moderately E-cadherin-expressing T24 2D4+ had a lower expansion rate.

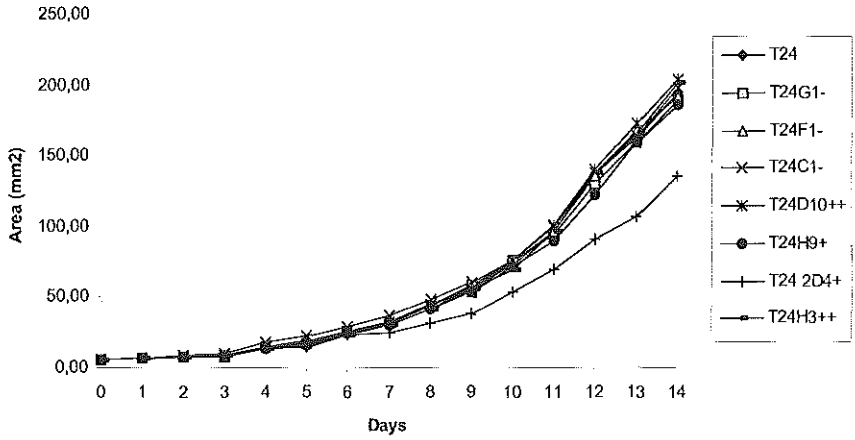

Figure 7: Expansion of T24 clones in the absence of surrounding normal urothelium. Data are expressed as means (n=4 cultures).

Discussion

The IEE assay used here is considered an experimental model relevant for the study of the mechanisms underlying the *in vivo* expansion of carcinoma *in situ* of the bladder, because in this model, IEE is the outcome of the balance between the regenerative potential of normal urothelial cells and the growth of transformed cells. Previous studies have shown that this *in vitro* IEE may be influenced by exposure to growth factors and by culture on substrates composed of particular extracellular matrix proteins (10,11). These earlier studies suggested that modulation of IEE was largely the consequence of the interaction of these factors with the normal urothelium, rather than a direct effect on the bladder carcinoma cells. The observed considerable variation among the six tested TCC cell lines in their capacity to expand at the expense of normal urothelium implies that factors intrinsic to these cell lines also determine the outcome of the cocultivations.

Previously, we have shown that the pattern of infiltration of TCC cells into the surrounding normal urothelium is determined by E-cadherin expression (10,11). E-cadherin negative TCC cells infiltrate the normal urothelium as individual cells, whereas tumor cells with a homogeneous expression of E-cadherin exhibit a sharp demarcation with the normal urothelium. The 1207 TCC cell line with a heterogeneous expression of E-cadherin *in vitro* also displays the capacity to infiltrate the normal urothelium as individual cells. Staining of these cocultivations with a human specific E-cadherin antibody revealed that the infiltrative single cells of the 1207 cell line had a reduced or absent expression of E-cadherin as compared with the high homogeneous expression of the primary tumor at the implantation site (data not shown). These results indicate that a reduced or absent expression of E-cadherin results in infiltration of the surrounding normal urothelium as single cells.

Comparison of the six TCC cell lines revealed that the two constitutively E-cadherin-negative bladder carcinoma cell lines (T24 and J82) hardly showed any IEE, whereas three of four E-cadherin-positive cell lines (SD, RT112, and 1207) did. We hypothesized that a functional E-cadherin-catenin complex is required for effective IEE of TCC cells. To further test this hypothesis, we established stable transfectants of T24 cells expressing the full-length mouse E-cadherin cDNA. Several clones of E-cadherin-expressing T24 cells were obtained. Functionality of the transfected T24 clones was analyzed with the embryonic chicken heart *in vitro* invasion assay (12,26) and a wound colonization assay (27).

The E-cadherin-negative cell line T24 was generally capable of invasion into the embryonic chicken heart, whereas only the T24 transfectants with high expression of E-cadherin (T24H3++, T24D10++) had a significantly reduced invasive capacity, implying a functional E-cadherin-catenin complex. The T24 clones with a moderate/low, but homogeneous expression of E-cadherin (*e.g.*, T24H9+) retained part of their invasiveness (Table 2). This observation is in accordance with those of *Vleminckx et al.* (12) who suggested that a threshold expression of E-cadherin has to be reached in order to prevent *in vitro* invasion into the embryonic chicken heart fragments. Similarly, in IEE assays a significant enhanced expansion rate was only observed for the two T24 clones with high E-cadherin expression but not for the T24 clones with a moderate or low expression level, suggesting that the same E-cadherin mediated mechanism induces both suppression of *in vitro* invasion and promotion of IEE. The enhanced E-cadherin-mediated expansion rate became manifest only under the cocultivations conditions of IEE. Thus, increased expression of E-cadherin by transfected T24 cells was neither associated with an increased expansion rate on collagen type IV-coated membranes nor with an increased proliferative activity or wound repair. Several reports documented E-cadherin-mediated suppression of cell motility and inhibition of proliferation (27-29). This discrepancy with our findings on T24 cells may be explained by the use of fibroblast cells in these studies rather than epithelial cells. Furthermore, the motility assays used in these reports (27-29) were based on single cell assays, whereas our expansion assay (Fig. 7) monitors the outgrowth of a cohesive sheet of cells.

Recently, Pignatelli (30) speculated on the possibility of a molecular cross-talk between cadherins and integrins in cancer cells. Because integrins are involved in motility and could be a regulator in IEE, we examined whether E-cadherin expression levels in transfected T24 cells would correlate with expression levels of integrins. Flow cytometric analysis of α1-, α2-, α3-, α4-, α5-, α6-, β1-, or β3-integrin expression levels did not point to such a mechanism in E-cadherin transfected T24 cells. It could still be argued that E-cadherins could lead to an altered localization or affinity of integrins on the cell membrane. Because we did not find any altered migratory behavior of the transfected T24 cells in our expansion assay in the absence of surrounding normal urothelium, E-cadherin-integrin cross-talk in our transfected T24 cells seems an unlikely mechanism for the observed IEE. We strongly feel that cohesion of transformed T24 cells is the most important E-cadherin-mediated determinant for IEE.

Our study suggests that E-cadherin is an important molecule for IEE of TCC *in vitro*, but it does not prove that this view holds true for bladder carcinoma *in situ*. Although a number of studies analyzed E-cadherin immunoreactivity during different stages of bladder cancer progression, none reported on the E-cadherin expression in carcinoma *in situ* of the bladder in detail (15-17,31). In a preliminary study, we stained paraffin sections with carcinoma *in situ* of ten patients for E-cadherin. All of these ten lesions had a normal, homogeneous, membranous expression of E-cadherin, confirming our hypothesis on the contributory role of E-cadherin in the intraepithelial propagation of bladder carcinomas.

For carcinogenesis *in vivo*, our observations would imply that on the one hand, E-cadherin promotes expansion of carcinoma *in situ* and on the other hand, opposes invasiveness of the transformed cells. Generalizing, E-cadherin-mediated cohesiveness may represent a major property of transformed clones, allowing carcinoma *in situ* to expand at the expense of surrounding normal epithelial cells *in vivo*.

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Chapter 5

INFLUENCE OF THE MICROENVIRONMENT ON INVASIVENESS OF HUMAN BLADDER CARCINOMA CELL LINES

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Abstract

To investigate the importance of the microenvironment in bladder cancer invasion, a panel of 6 bladder carcinoma cell lines (SD, RT112, JON, 1207, T24, J82) was tested both in *in vitro* and *in vivo* invasion assays. Furthermore, invasiveness was correlated with the expression of components of the E-cadherin-catenin complex.

The E-cadherin negative cell lines, T24 and J82, displayed a high *in vitro* invasive capacity, whereas the E-cadherin positive cell lines, SD and JON, completely lacked *in vitro* invasive capacity. In contrast, *in vivo* invasion was noted for all cell lines, with the exception of cell line JON. Most notably, SD formed highly invasive tumors *in vivo*. The *in vivo* invasiveness of the E-cadherin positive bladder carcinoma cell lines was associated with a heterogeneous expression of the E-cadherin-catenin complex. The discrepancy between *in vitro* and *in vivo* invasive behavior implies that *in vivo* the microenvironment plays an important role in the establishment of the invasive phenotype. In addition, it was found that orthotopic xenografting of 1207, respectively T24 bladder carcinoma cells resulted in site-specific tumor take, respectively an enhanced tumor outgrowth and invasiveness as compared to heterotopic (*i.e.* subcutaneous) inoculation.

We conclude that the site-specific growth and invasion of the bladder carcinoma cell lines *in vivo* and the observed assay specific invasion (*in vitro* vs. *in vivo*) points to an effect of the local (bladder) microenvironment on tumor cell behavior.

Introduction

Bladder cancer is the fifth most common malignancy in males in the western world. From a clinical point of view, bladder cancers can be divided in superficial and deeply invasive tumors. Superficial bladder carcinomas have a relative good prognosis, despite a high recurrence rate after transurethral resection (Kurth et al, 1989). Only a small proportion of these superficial carcinomas progress to invasive disease, and distant metastases only seldom develop (Liponnen, 1989). Invasive bladder carcinoma, however, has a much less favorable prognosis than superficial bladder carcinoma. Despite the use of aggressive therapies, invasive bladder carcinomas have a five-year survival rate of 50% or less (Raghavan et al, 1990). The key regulators involved in the invasion of bladder tumors into the bladder wall are only partially understood. A further elucidation of these processes requires relevant *in vitro* and *in vivo* tumor models.

A currently available *in vitro* model for tumor invasion is the well-documented embryonic chicken heart invasion assay, which has been used to study several epithelial tumor systems (Vleminckx et al, 1991; Behrens et al, 1993; Vermeulen et al, 1995). Since paracrine effects of the host tissue may regulate the expression of invasion promoting or suppressor molecules, it can be anticipated that *in vitro* observations may not necessarily be identical to *in vivo* findings. Furthermore, for some tumor systems it was demonstrated that the site of tumor cell inoculation might influence tumor growth, invasion and metastasis (Morikowa et al, 1988). Particularly, orthotopic xenografting has been shown to enhance tumor take, invasive properties and metastatic behavior of a number of human carcinoma cell lines (Naito et al, 1988; Rembrink et al, 1997). Therefore, we compared the *in vitro* invasive properties of six human bladder carcinoma cell lines with their invasiveness after orthotopic and heterotopic inoculation in SCID (severe combined immunodeficiency syndrome) mice. SCID mice were chosen as a host strain, since it was demonstrated that these mice were more apt to permit xenograft take than nude mice (Chang, 1966; Calaf et al, 1993; Jankun et al, 1997).

An initial step in the invasive process is the detachment of tumor cells from the in situ carcinoma. It has been proposed that loss of cell-cell adhesions is a prerequisite for detachment of cells from the primary tumor mass. Epithelial cell-cell adhesion is primarily determined by the E-cadherin-catenin complex. In tumor models, loss of E-cadherin expression appears to be associated with the gain of the invasive phenotype. Similarly, loss of the invasion suppressor molecule E-cadherin is predictive for poor survival of patients with

bladder cancer (Bringuier et al, 1993; Syrigos et al, 1995). Furthermore, abnormal expression of the cytoplasmatic, E-cadherin associated, proteins (α-,β-,γ- catenin and $p120^{cas}$) was also correlated with tumor grade, stage and poor prognosis (Shimazui et al, 1996). Because these studies suggest that the downregulation of the E-cadherin-catenin complex is involved in invasive bladder carcinoma, we also studied the expression of members of the E-cadherin-catenin complex during the *in vitro* and *in vivo* invasion assays.

Our results showed that the used transitional cell carcinoma (TCC) cell lines had different cell biological behavior in the applied bioassays. *In vitro*, invasion of the TCC cell lines into the embryonic chicken heart fragments was strongly correlated with absent or heterogeneous expression of E-cadherin. However, transplantation of tumor cells into SCID mice resulted in an enhanced invasive capacity of the E-cadherin positive TCC cell lines. Furthermore, two TCC cell lines (1207 and T24) exhibited site specific growth and invasion. These results point to a specific influence of the (bladder) microenvironment on tumor growth and invasion.

Material and Methods

Cell lines

The human bladder cancer cell lines T24, SD, RT112, JON and J82 were kindly provided by Prof. Dr. J.A. Schalken, Urological Research Laboratory, University Hospital Nijmegen, The Netherlands, and have been characterized previously (Paulie et al, 1983; Masters et al, 1986). Bladder carcinoma cell line 1207 was obtained from Dr. W.I. De Boer, GETU Service d'Urologie, Paris, France (De Boer et al, 1997). Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere.

Antibodies

Monoclonal antibody RCK108 directed against cytokeratin 19 (Eurodiagnostica, Arnhem, The Netherlands) or monoclonal antibody DC10 directed against cytokeratin 18 (Beckman Coulter, Fullerton, CA, USA) were used to distinguish the human bladder carcinoma cells from murine tissue (*in vivo* assay) or embryonic chicken heart tissue (*in vitro* assay). This is based on the species specificity of these two antibodies for human cytokeratins. Monoclonal antibody DC10 was used for staining J82 cells whereas the other cell lines were stained with monoclonal antibody RCK108. Nearly 100% of the bladder carcinoma cells of each cell line were labeled with RCK108 or DC10. The mouse monoclonal anti-bromodeoxyuridine (BrdU) antibody IIB5 (kindly donated by Dr. B. Schutte, University of Maastricht, The Netherlands) was used to visualize cells that had incorporated BrdU during the S-phase of the cell cycle. Expression of E-cadherin was demonstrated with the monoclonal antibody 5H9 (Eurodiagnostica), raised against an 80-kDa tryptic fragment of E-cadherin derived from human A-431 carcinoma cells. This antibody also detects the 120-kDa mature E-cadherin protein (Moll et al, 1993). The monoclonal antibodies against α- and β-catenin were obtained from Transduction Laboratories (Lexington, USA).

Immunohistochemistry

E-cadherin, α- and β-catenin expression by bladder tumor cells *in vitro* was determined as follows. Cell lines were cultured on multichamber slides until confluency was reached and fixed in cold acetone (-20°C) for 1 hour. After drying, the slides were incubated with the

different primary antibodies followed by visualization with FITC-conjugated rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark).

For immunohistochemistry on paraffin embedded tissues, five □m thick paraffin sections were mounted on 3-amino-propyl-tri-ethoxy-silane (Sigma, St. Louis, USA) coated slides and dried overnight at 37°C. Sections were deparaffinized, endogenous peroxidase was blocked in 3% H_2O_2 in methanol (20 minutes) and rinsed in PBS.

For antigen retrieval, sections were digested for 10 minutes in 0.1% pronase (Sigma) (containing 1% $CaCl_2$ for E-cadherin staining) in PBS. Prior to α- and β-catenin staining, sections were boiled twice for 5 minutes in a solution of 0.01 M citrate (pH 6.0) in a microwave oven. Paraffin sections of normal human skin were used as positive controls for E-cadherin, α- and β-catenin staining. For BrdU-staining, after the antigen retrieval procedure was performed, slides were incubated in 2N HCl for 30 minutes, followed by a two times 5 minutes wash with Borax buffer (Schutte et al, 1987). Prior to the application of the primary antibodies non-specific binding was blocked with 10% normal goat serum diluted in PBS containing 1% bovine serum albumin. Incubation with the primary antibody was followed by a biotinylated goat anti-mouse antibody (Dako) and subsequently a horseradish peroxidase conjugated streptavidin-biotin complex (Biogenex, San Ramon, USA). Peroxidase activity was visualized with 0.03% H_2O_2 and 0.02% 3,3,-diaminobenzidine tetrahydrochloride (DAB; Fluka, Basel, Switzerland) diluted in PBS. The slides were counterstained with Mayer's hematoxylin, dehydrated and mounted.

In vivo tumorigenicity of cell lines

The tumorigenicity of each cell line was tested in six weeks old female SCID mice (B17/ ICR Han Hsd-SCID), obtained from Harlan (Zeist, The Netherlands). Bladder carcinoma cells suspended in Hanks buffered salt solution were injected both subcutaneously (± 4 x 10^6 cells) in a volume of 200 µl and in the submucosa of the bladder (± 10^6 cells) in a volume of 50 µl. For inoculation of tumor cells in the bladder wall an abdominal incision was made in mice anaesthetized with avertine (0.25 mg/g body weight). One hour before the mice were sacrificed, they received an intraperitoneal injection of BrdU (40 mg/kg body weight) in 0.15 M NaCl. Under a dissecting microscope tumor inoculation sites plus other relevant organs were removed and fixed in 4% phosphate-buffered formalin for 16 hours before paraffin embedding.

Determination of the proliferative activity of TCC in vivo

Proliferative activity was determined by counting BrdU-positive nuclei in a total of 1000 nuclei, at four random areas of the tumor. Proliferation is expressed as the labeling index: Number of BrdU-positive nuclei/ total number of nuclei x 100%.

Morphometry

The size of *in vivo* formed tumors was estimated by image analysis. Serial sections were cut and the estimated largest tumor area was determined from the immunostainings with the RCK108 or DC10 antibody. Subsequently, these tumor areas were quantitated with a Hitachi CCTV camera equipped with the KS400 image analysis software package (Kontron Elektronik, Eching, Germany).

In vitro invasion assay

Chicken heart invasion assays were performed as described by Mareel *et al.* (1977). Briefly, human bladder tumor cells were confronted with precultured rounded fragments of embryonic chicken heart on soft agar for 24 hours. Next, the fragments with attached bladder tumor cells were kept in suspension culture under gyrotory shaking (120 rpm, 37°C) in MEM REGA 3 medium (GibcoBRL/Life Technologies, Breda, The Netherlands) containing 10% FCS. After 6 days of cocultivation, fragments were fixed in Bouin-Hollande's solution and embedded in paraffin. Invasion was scored on serial histological sections stained with hematoxylin/eosin. The tumor cells can be discerned from the heart tissue by their more basophilic staining. Additional confirmation of invasion was obtained by selective keratin (RCK108, DC10) immunostaining of the human bladder carcinoma cells.

Morphology of tumor cells on Matrigel

The morphological appearance of bladder tumor cells on Matrigel was assessed. Therefore, 200 µl growth factor reduced Matrigel (9.64 mg/ml; Becton Dickinson, Bedford, USA) per well was allowed to polymerize for 30 minutes at 37°C in a 24-wells plate. Subsequently, a same Matrigel solution containing 1 x 10^4 tumor cells was poured on top of the polymerized matrigel. After the gelation of the second Matrigel solution, standard culture medium was pipetted on top of it. After 1 week, morphology of the bladder tumor cells (epithelial or fibroblast-like) was recorded by microscopical examination.

Results

In vivo tumorigenicity

Tumor take, tumor size, muscle invasion and proliferative activity after heterotopic (subcutaneous) and orthotopic inoculation of each of the six cell lines was examined (Table I). A tumor take of 100% was observed for SD and RT112 bladder carcinoma cells both after orthotopic and heterotopic inoculation. Both cell lines clearly invaded the muscle tissue underlying the skin or bladder mucosa (Figure 1A, 1B). The size of the subcutaneous tumors was 4- to 10-fold larger as compared to the bladder tumors, possibly reflecting the larger number of cells inoculated subcutaneously. SD and RT112 tumors occasionally penetrated through the bladder mucosa into the bladder lumen. The normal mouse urothelium covering the tumor had often a hyperplastic appearance, which may be attributed to the paracrine action of tumor-derived growth factors (De Boer et al, 1994).

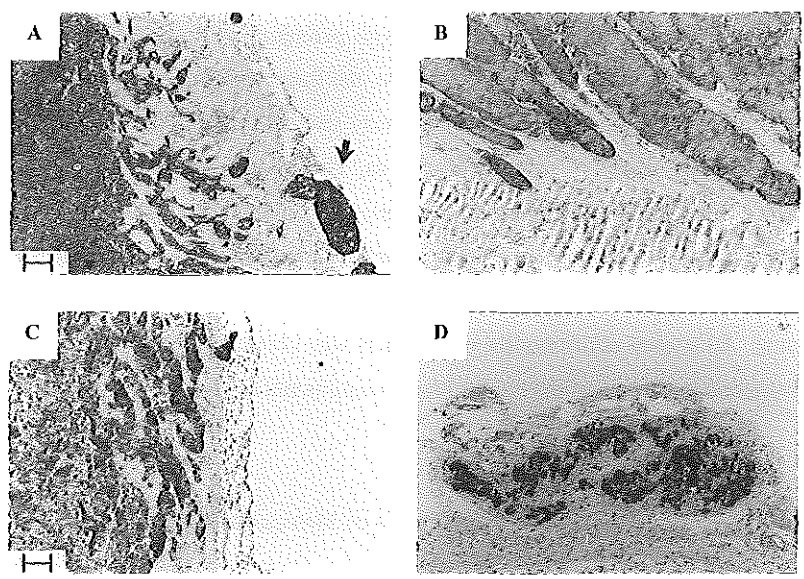

Figure 1: Tumorigenicity of bladder tumor cells. Tumor cells were selectively stained with RCK108. Invasion of RT112 cells into the submucosa and muscularis of the bladder (A) and the skeletal muscle underneath the skin (B). Note the clump of RT112 cells in a perivesicular lymph vessel of the bladder (Fig. 1A; arrow). Part of a large orthotopic tumor of T24 cells in the bladder (C), and as a reference the small tumor in the subcutis (D). Scale bar = 100μm.

Table I: HETEROTOPIC AND ORTHOTOPIC TUMORIGENICITY OF BLADDER CARCINOMA CELL LINES

Cell line	Mean follow-up period in days (range)	Bladder		Subcutaneous		E-cadherin *in vivo*[a]	Labeling Index[b]		Estimated largest area	
		tumor take	muscle invasion	tumor take	muscle invasion		bladder mean±stdev	subcutaneous mean±stdev	bladder mean±stdev	subcutaneous mean±stdev
SD	39.5 (35-53)	4/4	4/4	4/4	4/4	Htr.	13.4±9.3	16.3±3.4	6.0±6.5	21.2±10.8*
RT112	37.5 (27-53)	4/4	3/4	4/4	3/4	Htr.	22.0±1.6	19.4	3.5±4.1	34.6±19.3*
T24	58 (35-74)	4/4	3/4	3/4	0/3	Neg.	10.3±2.9*	2.0±0.7	4.0±1.1*	1.2±1.6
1207	50.6 (29-53)	4/5	2/4	0/5	0/0	Htr.	16.1±3.8	[c]	2.2±2.6	[c]
Jon	50.2 (31-57)	5/5	0/5	4/5	0/4	Hom.	16.3±2.6	13.2±4.3	1.5±1.5	2.6±1.6
J82	42 (40-53)	2/5	0/2	1/5	1/1	Neg.	21.0±0.0	17.3[d]	3.1±4.1	53.2

a) Htr.: heterogeneous, Hom.: homogeneous, Neg.: absent expression at the cell membrane; b)labeling index: percentage of BrdU positive cells;
c) no subcutaneous tumors; * = significantly different in bladder as compared to subcutaneous (t-test; $P<0.05$).

We observed a high take rate after both heterotopic and orthotopic inoculation of T24 cells in SCID mice (Table I). Strikingly, subcutaneous T24 tumors were rather small and no muscle invasion was noted (Table I, Figure 1D). In contrast, T24 tumors, localized in the bladder, were about 3-fold larger, showed muscle invasion (Figure 1C) and had a significantly higher proliferative activity as compared to subcutaneous T24 tumors (Table I).

Site specific tumor take was seen for cell line 1207. Although in 4 out of 5 mice, orthotopically injected with 1207 cells, tumors had developed in the bladder wall (2 were muscle invasive), no tumors were found at the subcutaneous injection sites despite of the larger number of subcutaneously inoculated tumor cells. Microscopic examination of the subcutaneous injection sites confirmed this observation. The two muscle invasive 1207 tumors also showed penetration into the bladder lumen.

JON cells demonstrated a high take rate both hetero- and orthotopically, but muscle invasion was completely lacking. The subcutaneous JON tumors showed an expanding growth pattern with sharp tumor borders. These tumors were associated with an inflammatory infiltrate. The tumor take of J82 was low, both after orthotopic and heterotopic inoculation. In one mouse a large muscle invasive subcutaneous tumor was found.

In none of the injected mice lymphogenic or distant metastases were found, but regional lymph vessels in the loose connective tissue surrounding the bladders of mice injected with SD, RT112 or T24 bladder carcinoma cells occasionally contained isolated small tumor aggregates (Fig1A).

In vitro embryonic chicken heart invasion assay and tumor cell morphology on Matrigel

In vitro invasion of bladder carcinoma cells was examined after 4-6 days of cocultivation with embryonic chicken heart fragments. Adhesion of tumor cells to chicken heart fragments varied from 53% of the cases for JON and J82 cells to 97% of the cases for the SD cells (Table II). T24 and J82 cells showed the strongest invasive capacity. After successful adhesion nearly all embryonic chicken heart fragments were infiltrated by these E-cadherin negative tumor cells (Figure 2A). RT112 and 1207 (Figure 2B) cells had a moderate capacity (33% and 39% of the cases, respectively) to invade heart fragments, whereas SD (Figure 2C) and JON (Figure 2D) cells were not able to invade the embryonic chicken heart fragments at all. The SD cell line formed a clear epithelioid cell layer around the embryonic heart tissue, a feature characteristic for cells with a functionally intact E-cadherin-catenin complex (Vermeulen et al, 1995).

To determine the epithelial or mesenchymal morphotype of the used cell lines, cell lines were cultured in solid Matrigel substrate (method adapted from Vermeulen *et al.*, 1995). After one week of culture in solid Matrigel (Table II) the constitutively E-cadherin negative T24 and J82 (Fig 2E) cells had a fibroblastic morphology. In contrast, the E-cadherin positive cell lines SD (Fig 2F), RT112 and JON expressed an epithelial morphology with formation of densely packed colonies. Bladder carcinoma cell line 1207 with a heterogeneous expression of E-cadherin, had an epithelial morphology when cultured in Matrigel.

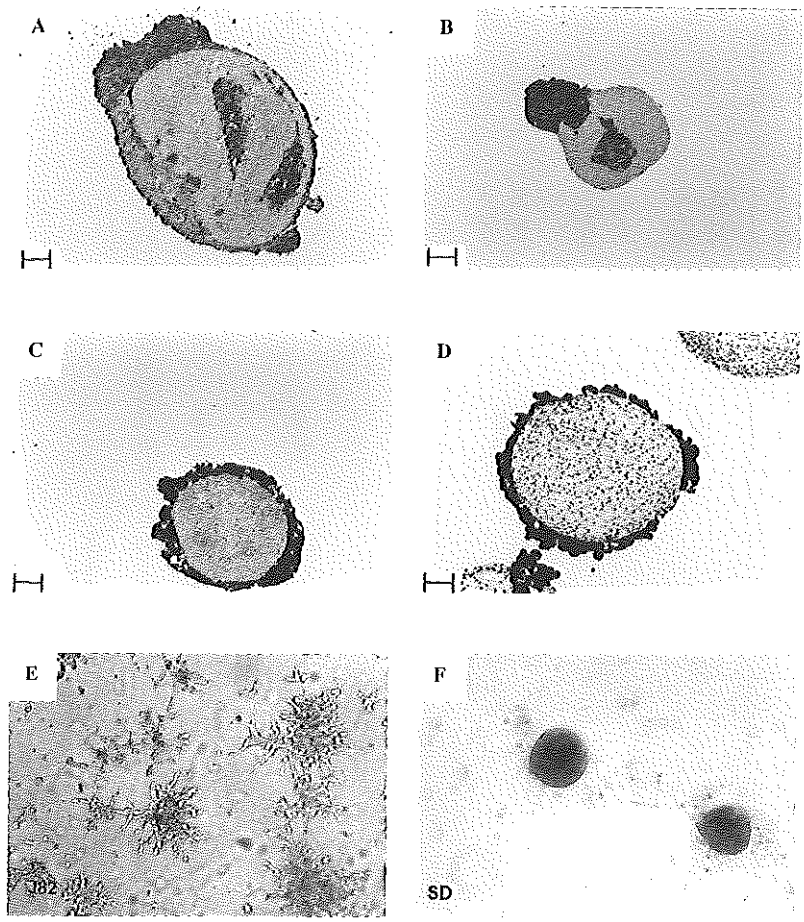

Figure 2: Confronting cultures of invasive and noninvasive TCC cells with embryonic chicken heart. Photographs of paraffin sections of embryonic chick heart fragments confronted in organ culture with T24 (A), 1207 (B), SD (C) or JON (D) cells. Bladder tumor cells were selectively stained with RCK108. Scale bar = 100μm, except Figure 2D scale bar = 50μm. Phase contrast pictures of J82 (E) and SD (F) cells cultured in solid Matrigel.

TABLE II: *IN VITRO* INVASION OF TCC CELL LINES

Cell line	E-cadherin[1] expression *in vitro*	α-catenin expression *in vitro*	β-catenin expression *in vitro*	*In vitro* confronting cultures	*In vitro* confronting cultures	Morphology Matrigel[5]
				Adherent[3]	Invasive[4]	
SD	Hom.	Hom.	Hom.	37/38 (97%)	0/37 (0%)	TC Epithelial
RT112	Hom.	Hom.	Hom.	9/15 (60%)	3/9 (33%)	TC Epithelial
T24	Neg.	Htr. Cytopl./cell-membr.[2]	Hom.	18/30 (60%)	16/18 (89%)	I Fibroblastic
1207	Htr.	Hom.	Hom.	28/45 (62%)	11/28 (39%)	TC epithelial
JON	Hom.	Hom.	Hom.	8/15 (53%)	0/8 (0%)	TC Epithelial
J82	Neg.	Htr. Cytopl./cell-membr.	Hom.	9/17 (53%)	8/9 (89%)	I Fibroblastic

1) Abbreviations as in table I.
2) cytopl./cell-membr. = cytoplasm/cell membrane.
3) Number of adherent / total number of confronted embryonic chicken heart cultures.
4) Number of invasive / number of confronted cultures with adherent tumor cells.
5) I=invasive, TC=tight colonies (criteria adapted from Vermeulen *et al.*).

In vitro and in vivo expression of E-cadherin and α- and β-catenin

Data on expression of E-cadherin and α- and β-catenin on confluent cultures of bladder carcinoma cell lines visualized by immunohistochemistry are summarized in Table II. Expression patterns of E-cadherin were compared between the *in vivo* and *in vitro* assays. Cell lines T24 and J82 lacked E-cadherin expression *in vivo* and *in vitro* (Table I & II). The cell lines SD and RT112 which display a homogeneous E-cadherin expression *in vitro* displayed a heterogeneous cell membrane immunostaining *in vivo* both in the subcutaneous tumors and in the tumors located in the bladder wall. Regions with normal expression of E-cadherin were seen in the central areas of these tumors (Fig. 3A). Reduced expression of E-cadherin was primarily seen in the invasive borders of these tumors (Fig 3B). Strikingly, the isolated small aggregates of SD and RT112 tumor cells in lymph vessels had a normal expression of E-cadherin (Figure 3C). The expression patterns of either α- or β-catenin was similar to the expression pattern of E-cadherin in the different tumors.

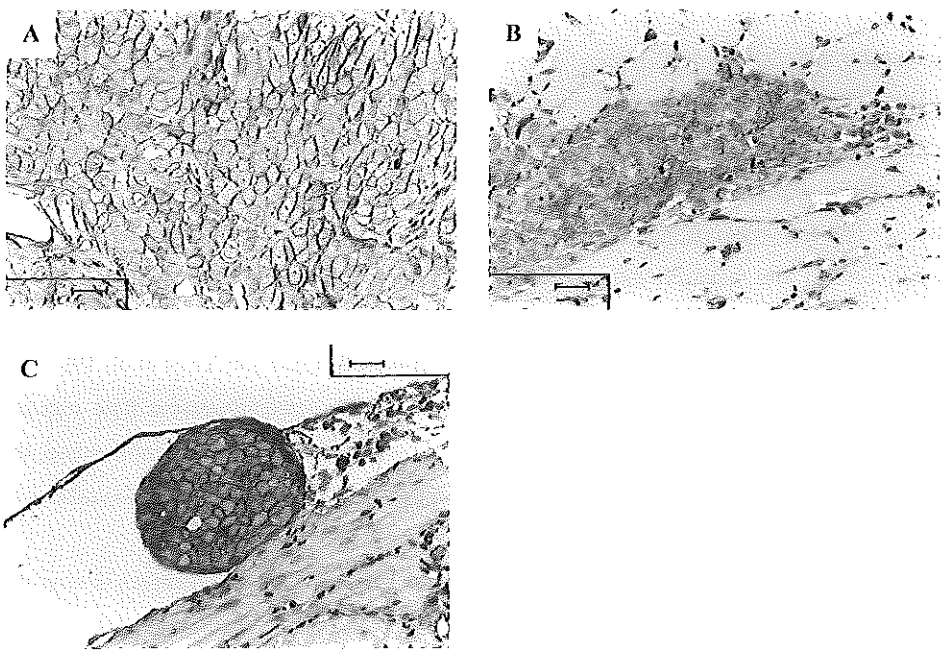

Figure 3: Expression of E-cadherin in vivo. Normal expression of E-cadherin in a SD tumor (A). Reduced expression of E-cadherin in an invasive region of a subcutaneous SD tumor (B). RT112 cells in a perivescular lymph vessel of the bladder, with normal expression of E-cadherin (C). Scale bar = 25μm.

Discussion

The six human bladder carcinoma cell lines studied in this paper display a considerable heterogeneity of cell biological characteristics with respect to their invasive potential both *in vitro* and *in vivo*.

SCID mice proved to be a very suitable host strain allowing successful grafting of all six examined human bladder carcinoma cell lines. This was emphasized by xenografting T24 cells in SCID mice. Tumor take of T24 cells in nude mice is poor or absent both after orthotopic and heterotopic inoculation (Masters et al, 1986; own observations). Strikingly, T24 tumor growth, as reflected by tumor size and proliferative activity as well as tumor invasiveness, was enhanced by the orthotopic inoculation (Table I). 1207 cells exhibited site-specific outgrowth, since 1207 tumor take only occurred after orthotopic transplantation. No signs of distant metastases for any of the xenografted TCC cell lines were found. Although isolated (RT112, SD, T24) tumor aggregates were found in the perivesicular loose connective tissue, no lymph node metastases were found. The short follow-up period of 2 months may account for this.

We compared the *in vivo* invasiveness of the various TCC cell lines with the results of the *in vitro* assay. The results of the *in vitro* embryonic chicken heart invasion assay did not match the *in vivo* invasive behavior of some of the tested TCC cell lines, most notably SD. The SD cell line was not invasive *in vitro*, but both after subcutaneous injection and after inoculation in the bladder wall SD cells developed muscle invasive tumors. Similar discrepancies between the embryonic chicken heart invasion assay and *in vivo* invasiveness were reported earlier in a study on colorectal carcinoma cell lines (De Vries et al, 1995; De Both et al, 1999). Our results support the view that the microenvironment may regulate invasive behavior of bladder carcinoma cells *in vivo*, for instance by influencing the expression of E-cadherin as suggested by Mareel *et al.* (1991), or by induction of synthesis of extracellular matrix degrading proteins.

We studied the immunochemical expression of the E-cadherin-catenin complex *in vivo* and *in vitro* and its correlation with invasiveness. The observed *in vitro* expression of the various members of the E-cadherin-catenin complex in the TCC cell lines was confirmed by western blotting by Giroldi *et al.* (1999). *In vivo*, a heterogeneous E-cadherin and catenin expression by SD and RT112 cells was observed, in contrast to their homogeneous E-cadherin-catenin expression *in vitro*. Loss or reduced expression of the E-cadherin-catenin complex was

primarily seen in the invasive regions of these tumors. These findings as well as the normal E-cadherin and α- and β-catenin expression by small aggregates of SD and RT112 tumor cells in lymph vessels (early sign of metastasis) suggests that the microenvironment may transiently reduce the expression of the E-cadherin-catenin complex during formation of invasive bladder tumors in SCID mice. Transient downregulation of E-cadherin expression *in vitro* is described previously and could possibly be accomplished through activation of exogenous *c-fos* (Reichmann *et al.*, 1992), via c-erbB2 (D'Souza and Taylor-Papadimitriou, 1994) or TGFβ (Mietinnen *et al.*, 1994). Furthermore, expression could be regulated by methylation of the E-cadherin gene or via tissue-specific responsive elements in the E-cadherin promoter (Christofori and Semb, 1999). Recent investigations have identified the transcription factor Snail, which could bind to E-boxes in the human E-cadherin promoter and thereby represses transcription of E-cadherin (Battle *et al.*, 2000; Cano *et al.*, 2000). Furthermore, Keirsebilck *et al.* described that the *in vivo* transient downregulation of E-cadherin could be caused by instability of the E-cadherin mRNA (Keirsebilck et al, 1998). Recently, Bringuier *et al.* showed that abnormal E-cadherin immunoreactivity in bladder tumors is associated with mRNA-downregulation or post-transcriptional downregulation of E-cadherin. In the same series of bladder tumors, no structural alterations of the E-cadherin gene were detected (Bringuier *et al.*, 1999). These findings corroborate with our observations on the transient reduction of E-cadherin expression in invasive bladder tumors.

A strong correlation was found between the *in vitro* expression of E-cadherin of bladder carcinoma cells and their ability to invade embryonic chicken heart fragments. Similar results were reported for cell lines of other origin (Vleminckx et al, 1991; Behrens et al, 1993; Vermeulen et al, 1995). The two E-cadherin negative cell lines T24 and J82 were generally capable of invasion of the embryonic chicken heart, whereas the (*in vitro*) homogeneously E-cadherin positive SD and JON cells showed no invasion. The epithelial morphology of these E-cadherin positive cell lines grown on Matrigel is in accordance with their functionally intact membrane bound E-cadherin-catenin complex (Vermeulen et al, 1995). On the other hand, the *in vitro* invasive cell lines, T24 and J82, had a fibroblastic morphotype when cultured on solid Matrigel. This is in accordance with the observation that epithelial-to-mesenchymal transition is correlated with gain of motility and invasive disease (Hay, 1995). The 1207 cells with a heterogeneous expression of E-cadherin had a rather limited infiltrative capacity in the embryonic chicken heart invasion assay. Surprisingly, RT112 cells with *in vitro* homogeneous E-cadherin and catenin expression were also capable of embryonic chicken heart invasion in

one third of the samples. Booth *et al.* showed that RT112 cells could invade the subepithelial capillary bed after culture on urinary tract stroma. In the same paper they showed that these RT112 cells had partially lost their E-cadherin expression (1997). A similar *in vitro* mechanism could have occurred in our embryonic chicken heart invasion assay.

In conclusion, we have studied the invasive properties of six bladder carcinoma cell lines, with the use of *in vitro* and *in vivo* invasion assays. We have shown that the use of SCID mice allows a high tumor take for bladder cancer cell lines, including those which proved to be poorly if at all tumorigenic in nude mice. On the basis of their site dependent tumor take, respectively tumor outgrowth, bladder tumor cell lines 1207 and T24 may be considered to resemble more closely organ confined human bladder carcinomas with regard to their response to growth modulating factors and extracellular matrix proteins. The view that the microenvironment influences the induction of bladder tumor invasion is supported by the observation that the results of the *in vitro* invasion assay of E-cadherin positive bladder carcinoma cell lines did not correlate well with their invasive properties *in vivo*. Transient downregulation of the E-cadherin-catenin complex may in part explain the observed invasive capacity *in vivo* of the latter cell lines.

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Chapter 6

A NEW *IN VIVO* MODEL OF CARCINOMA *IN SITU* OF THE URINARY BLADDER

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Abstract

Carcinoma in situ (CIS) of the urinary bladder is a neoplasm with an uncertain clinical behavior. It is either detected as an isolated disease or it is found in association with papillary or invasive transitional cell carcinoma (TCC). Results from mapping studies, follow-up studies and genetic analyses gave evidence that CIS can be regarded as a precursor lesion for invasive bladder carcinoma. Relevant in vitro and in vivo models are needed to identify factors that are involved in the growth and progression of CIS. To establish an in vivo model of CIS, cells of various TCC cell lines were intraluminally injected immediately after partial denudation of mouse bladders. All cell lines carried a p53 mutation associated with immunohistochemical overexpression of p53. The denuded bladders are rapidly reepithelialized with mouse urothelial cells and the injected tumor cells. By this procedure CIS lesions were observed with all used TCC cell lines. Cell line 1207 showed the highest frequency of CIS formation (70%) in the bladder. Next, the growth of the 1207-derived CIS was studied during six months. In this experiment 97% of the mice developed CIS-lesions. During the six months period we noted a steadily increase in the growth of the 1207-derived CIS, as was confirmed by high proliferation and low apoptosis of the tumor cells at all time-points. Furthermore, we could demonstrate that 1207-derived CIS shows signs of intraepithelial pagetoid spread of small clusters or single tumor cells. Only in two mice, besides formation of CIS, (limited) invasion was noted. This demonstrates that progression to invasive disease during the 6 months follow-up period is uncommon in this model. Importantly, this model provides evidence that an otherwise invasive tumor cell line loses its invasive potential as a consequence of implantation on the basement membrane.

The established in vivo model of 1207-derived CIS in denuded mouse bladder is useful in the study of the growth of CIS. The described model can be used to design future intravesical chemo-, immuno- or gene-therapies for CIS of the bladder.

Introduction

Urinary bladder cancer is the fifth most common malignancy in males in Western societies. At initial diagnosis, the majority (70-80%) of these bladder tumors concerns superficial, non-invasive transitional cell carcinomas (TCC). After treatment by transurethral resection, the patients presenting with superficial bladder cancer have a high risk of tumor recurrence (± 70%), which can ultimately lead to invasive disease[1]. Only approximately 20-30% of the TCC emerges as deeply invasive tumors that penetrate the basement membrane and show invasion into the underlying tissue of the bladder wall. Despite radical therapies, like cystectomy, the clinical outcome of these invasive tumors is poor[2].

Many papers report that high-grade TCC are associated with the occurrence of carcinoma in situ (CIS). CIS is characterized by the intraepithelial presence of dysplastic cells, without penetration of the underlying basement membrane. The occurrence of CIS in the flat urothelium adjacent to tumors is correlated with a higher probability of tumor recurrences and/or invasion[3,4]. The clinical course of CIS without accompanying invasive carcinoma is highly variable, but without treatment more than 50% progresses to invasive tumors[5]. On the other hand, a significant minority of patients with isolated CIS will never have progression to invasive disease[6,7]. Although this variation in biological behavior of CIS clearly exists, CIS is generally regarded as the precursor lesion for invasive carcinoma. First evidence for a preexisting CIS phase before the onset of invasive carcinoma came from mapping studies of cystectomy specimens[6-9] and from follow-up studies[10]. Furthermore, mutation and loss of heterozygosity (LOH) analyses have shown that CIS displays the same genetic alterations as invasive bladder tumors[11,12]. The above results suggest that CIS is indeed the precursor lesion for invasive bladder carcinomas, but additional factors determine if progression occurs. To date, several factors are known to be associated with the progression of CIS to invasive disease. These include diffuse growth, prostatic involvement, overexpression of p53 or proliferation-, surface- or tumor-associated markers and loss of normal urothelial antigens[13].

Although CIS is regarded as a precursor for invasive bladder carcinoma relevant in vivo and in vitro models of CIS are rare. Previously, we have established an in vitro model for the study of the intraepithelial expansion of bladder tumor cells[14]. We showed that some human TCC cell lines were able to expand by replacing the normal urothelium. To develop an in vivo model of CIS, we inoculated tumor cells on partially denuded mouse bladders. The

mouse bladders were denuded to obtain a higher take rate and subsequent growth of inoculated bladder tumor cells. The denuded bladders are rapidly repopulated with mouse urothelial cells and the injected human tumor cells. Earlier, Soloway and Masters have shown that cauterization of the bladder wall prior to insertion of tumor cells resulted in an enhanced take rate[15]. In our study, the intraluminal injection of the TCC cell lines in denuded mouse bladders resulted in some cases in the development of lesions within the bladder which have the characteristics of CIS. Because TCC cell line 1207 gave the best results with respect to formation of CIS, we further determined the growth kinetics of the intraepithelial tumors of this 1207 cell line.

Our in vivo model represents a useful tool for the study of the mechanisms that underlie the growth and progression of CIS. Future therapies that prevent the expansion and progression of CIS to invasive tumors can also be investigated in the described model.

Material and Methods

Cell lines

The human bladder cancer cell lines T24, SD, RT112 were kindly provided by Prof. Dr. J.A. Schalken, Urological Research Laboratory, University Hospital Nijmegen, The Netherlands. Human bladder carcinoma cell line 1207 was obtained from Dr. W.I. De Boer, GETU Service d'Urologie, Paris, France[16]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in humidified atmosphere with 5% CO_2.

Denudation of bladder urothelium and subsequent inoculation of bladder carcinoma cells

Six weeks old female SCID mice (B17/ICR Han Hsd-SCID; Harlan, Zeist, The Netherlands) were anaesthetized with avertine (250 mg/kg body weight). The bladder was distended by intravesical injection of 200 µl PBS via a catheter (0.28 mm inner and 0.61 mm outer diameter polythene tube; Portex, Hythe, United Kingdom). This overstretching was maintained for 5 minutes. The presence of blood in the urine was regarded as an indication of damage to the urinary bladder. Immediately after damage, a suspension of bladder tumor cells (2×10^6 cells in 100 µl Hanks buffered salt solution [HBSS]) was injected into the lumen of the bladder via the catheter. Voiding of urine was prevented for 10 minutes by clamping off the external urinary tract of the mouse. Mice were sacrificed between one and two months after the procedure. Thirty minutes before the mice were sacrificed, they received an intraperitoneal injection of 5-bromo-2'-deoxyuridine (BrdU) (40 mg/kg body weight) in PBS. In the first experiment, comparing four different TCC cell lines, a total body autopsy was performed and organs were fixed in 4% phosphate buffered formalin for 16 hours before embedding in paraffin. On basis of the results of the first experiment, the second experiment was performed under milder conditions of overstretching followed by inoculation of 1207 cells only. Mice were sacrificed at various time points after inoculation of the 1207 tumor cells. In these experiments, the whole urinary tract including the regional lymph nodes was isolated.

The inoculated human bladder tumor cells were identified with a human specific cytokeratin 19 antibody, which lacks crossreactivity with mouse tissue. In this study, a tumor was scored as CIS when the tumor cells were confined to the urothelium, without visual

penetration of the basement membrane.

In an additional experiment, PBS with the addition of Indian ink was used to distend the bladder, while omitting the subsequent inoculation of TCC cells. The Indian ink selectively attached to areas with total denudation of the urothelium. In this manner, the extent of denudation of the bladder urothelium could be visualized.

Antibodies

The following antibodies were used: mouse monoclonal antibody RCK108 directed against human cytokeratin 19 (Eurodiagnostica, Arnhem, NL); mouse monoclonal antibody to bromodeoxyuridine (BrdU), clone IIB5 (kindly donated by Dr. B. Schutte, University of Maastricht, NL), mouse monoclonal antibody to p53 (DO-7; DAKO, Glostrup, Denmark) and rabbit polyclonal antibody to collagen type IV (Eurodiagnostica).

Immunohistochemistry

For immunohistochemistry on paraffin embedded tissues, four µm thick paraffin sections were mounted on 3-amino-propyl-tri-ethoxy-silane (Sigma, St. Louis, USA) coated slides and dried overnight at 37°C. Sections were deparaffinized, endogenous peroxidase was blocked in 0.3% H_2O_2 in methanol (20 minutes) and rinsed in PBS. For antigen retrieval, sections were digested for 10 minutes in 0.1% pronase (Sigma) in PBS. After the antigen retrieval procedure, BrdU-staining was performed by incubating the slides in 2N HCl for 30 minutes at 37°C, followed by a two times five minutes incubation with Borax buffer (pH 8.5) and three times five minutes wash in PBS[17]. In the case of collagen type IV staining antigen retrieval consisted of incubation in 0.4% pepsin (Sigma) in PBS for two hours at room temperature. Prior to the application of the primary antibodies non-specific binding was blocked with 5% non-fat dry milk diluted in PBS containing 5% bovine serum albumin. Overnight incubation at 4°C with the primary antibody was followed by a biotinylated goat anti-mouse antibody (DAKO) and subsequently a horseradish peroxidase-conjugated streptavidin-biotin complex or an alkaline phosphatase-conjugated streptavidin-biotin complex (Biogenex, San Ramon, USA). As chromogen we used 3,3,-diaminobenzidine tetrahydrochloride (DAB) (Fluka, Basel, Switzerland), or New Fuchsin (Sigma). The slides were counterstained with Mayer's hematoxylin, dehydrated and mounted.

Analysis of proliferation and apoptosis

Sections were double stained with the BrdU/RCK108 antibodies. The double staining procedure consisted of two sequentially performed streptavidin-biotin complex methods as described in the immunohistochemistry section. The streptavidin-biotin complex reactive with the BrdU antibody was horseradish-peroxidase labeled, whereas the second complex reactive with the RCK108 antibody was alkaline-phosphatase labeled. Proliferative activity of CIS (RCK108 positive cells) was determined by counting BrdU positive nuclei in at least 100 tumor cells. Proliferation is expressed as the labeling index: Number of BrdU positive nuclei/ total number of nuclei x 100%. By morphological criteria we determined the number of apoptotic nuclei and expressed them as percentage of the total number of nuclei present in the CIS lesions (Apoptotic Index).

Extent of CIS in mice inoculated with cell line 1207

We semi-quantitatively determined the extent of CIS in mice inoculated with the TCC cell line 1207. To this end, the bladders of these mice were sectioned completely. With 20-section intervals (80μm), presence of 1207 cells was examined by cytokeratin 19 staining. Approximately 15 to 20 levels per urinary bladder were examined by this procedure. In these sections, we determined the percentage of the basement membrane that was covered with CIS (BMC). Furthermore, we determined the time-course of CIS formation at early time-points (14 days to 2 months) by recording the number of cytokeratin 19 positive single cells, groups of 2-5 cells, 5-10 cells or more than 10 cells.

P53 mutation analysis

From *in vitro* cultured TCC cells DNA was isolated using standard phenol-chloroform extraction. Exons 5 – 8 from the p53 gene are each amplified with published primers[18]. PCR was performed with 1-3 μl isolated DNA in a final reaction volume of 15 μl containing: 1.5 mM $MgCl_2$, 0.02 mM dATP, 0.2 mM dGTP, dTTP and dCTP each, 0.8 μCi α-^{32}PdATP (Amersham, Buckinghamshire, UK), 20 pmol of each primer and 0.2 unit *Taq* polymerase (Promega, Madison, WI, USA). PCR was performed for 35 cycles (denaturing at 95°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min) in a Biometra thermocycler. A final extension was carried out at 72°C for 10 min. PCR products were diluted with loading buffer (95% formamide, 10 mM EDTA (pH 8.0), 0.025% bromophenol blue and 0.025%

xylene cyanol), denatured at 95°C for 4 min and snap-cooled on ice. For SSCP analysis the samples were run overnight at 7W on a non–denaturing 6% polyacrylamide gel containing 10% glycerol in 1 x TBE running buffer. After electrophoresis, gels were fixed in 10% acetic acid, dried on blotting paper on a vacuum gel dryer and exposed to X-ray film overnight at –70°C, using intensifying screens. Films were evaluated by visual inspection.

Results

Establishment of CIS and invasive carcinoma in denuded mouse bladder

Overstretching of the mouse bladder resulted in detachment of urothelial cells with focal loss of the total urothelial lining, areas with one epithelial cell layer and areas with no detectable loss of urothelium. Collagen type IV staining showed that the basement membrane was preserved in the areas where the urothelium had detached (Figure 1A). Earlier experiments have shown that within five days after denudation the bladder wall was completely repithelialized[19]. Here we noted attachment of TCC cells to the damaged areas in the urinary bladders at one hour after the inoculation (Figure 1B).

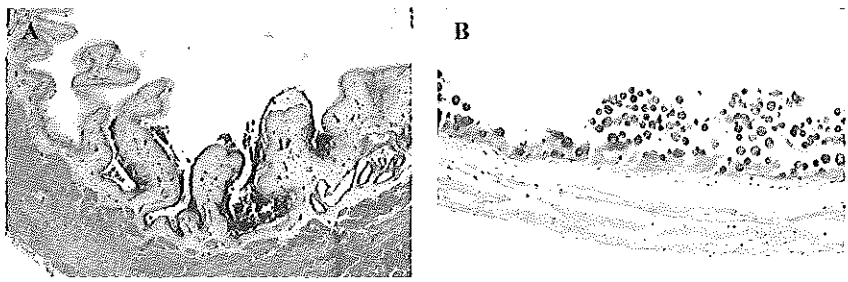

Figure 1: Short-term effects of overstretching of the mouse bladder and subsequent inoculation of TCC cells. A) Partial denudation of mouse bladder urothelium one hour after overstretching without inoculation of TCC cells. Areas with total denudation of the urothelium are highlighted by the selective attachment of Indian ink (black). Note that the basement membrane as visualized by Collagen type IV (brown) is preserved after denudation; magnification 100x. B) Inoculation of TCC cells on denuded bladder wall: attachment of RCK108-stained 1207 cells (brown) to damaged areas in the urothelium at 1 hour; 200x.

Inoculation of TCC cells in partially denuded bladder results in formation of CIS and/or invasive tumors in the bladder at 1 to 2 months (Table 1). However, there was a considerable heterogeneity among the various cell lines in tumor take, growth and localization in the urinary tract. Take rate in the bladder varied between 100% (1207) and 63% (RT112). Formation of CIS varied among the different cell lines from 38% to 70%. For RT112 and especially T24 mainly single tumor cells (Figure 2A) or small clusters of tumor cells were present in the urothelium, with a low proliferative activity and high number of tumor cells with morphological signs of apoptosis (data not shown). On the other hand, large areas of lining urothelium were replaced by either SD or 1207 cells (Figure 2B-C). Staining with collagen type IV antibody confirmed that the CIS areas were really confined to the urothelium and had not penetrated the basement membrane (Figure 2D).

Table 1. Take Rate and Growth of Inoculated TCC Cell Lines after Partial Denudation of the Urinary Bladder of SCID Mice

Cell line	Number of mice	TCC implants in the urinary tract	Bladder			Kidney		TCC implants in the ducts of the clitoral gland (%)
			Total (%)	Invasive (%)	CIS (%)	PN[1]	Tumors (%)	
SD	7	7/7	6/7 (86%)	2/7 (29%)	4/7 (57%)	7/7	6/7 (86%)	6/7 (86%)
T24	7	7/7	6/7 (86%)	1/7 (14%)	5/7* (71%)	5/7	4/7 (57%)	2/7 (29%)
RT112	8	7/8	5/8 (63%)	2/8 (25%)	3/8* (38%)	6/8	5/8 (63%)	5/8 (63%)
1207	10	10/10	10/10 (100%)	3/10 (30%)	7/10 (70%)	9/10	0/10 (0%)	5/10 (50%)

1) PN = pyelo-nefritis; * = mainly single cells in the urothelium.

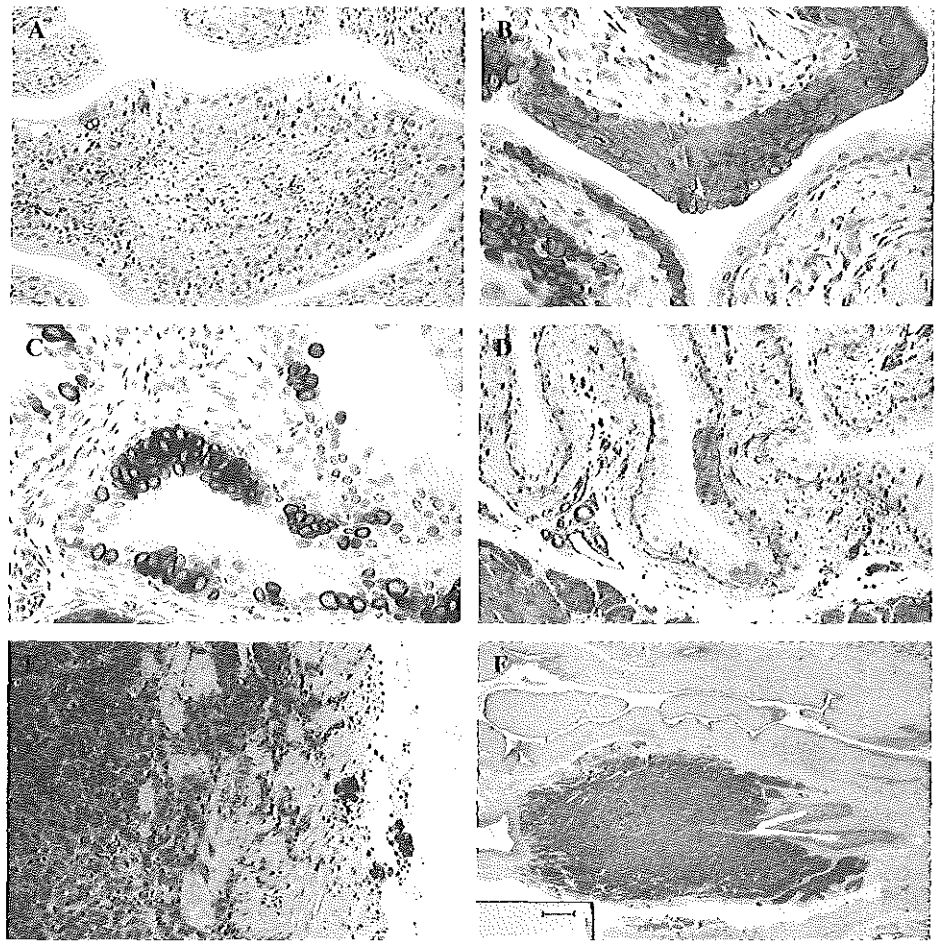

Figure 2: Tumor implants in the urinary tract of the SCID mouse. A) Single RCK108-positive T24 cells (brown) in the urothelium of the mouse; magnification 200x. B) Formation of CIS after inoculation of SD and C) 1207 cells in denuded bladder; 400x. D) Section with 1207-derived CIS double stained with RCK108 (red) and Collagen type IV (brown) antibody to confirm that the tumor area is confined to the urothelium; 200x. E) Detail of a SD-derived muscle invasive tumor in the bladder; 200x. F) SD tumor in the ducts of the clitoral gland; 50x.

Although the SD cell line forms CIS in 57% of the mice, in two mice (29%) highly invasive bladder tumors (Figure 2E) were found. Also in the case of 1207 superficially invasive bladder tumors (30% of the cases) were detected, additional to areas with CIS.

Strikingly, TCC implants were found repeatedly in the ducts of the clitoral gland, which empty into the urethra (Figure 2F). These implants are probably the result of mechanical damage to that area by the catheter. Furthermore, a high percentage of tumor involvement in the kidney often accompanied by pyelo-nefritis was noted, which could be the result of reflux of urine containing tumor cells at the beginning of the experiment. No distant metastases were found in any of the mice, but in mice inoculated with SD or RT112 cells local metastases were found in the regional lymph vessels of the bladder and the kidney. The occurrence of these local metastases was always accompanied by invasive tumors in either the bladder or the kidney.

From this experiment, we concluded that inoculation of 1207 cells in denuded mouse bladder is the most promising model for the study of CIS, because it had the highest frequency of CIS in the bladder, and long term experiments were less likely to be hampered by mortality due to tumor spread.

P53 mutation analysis

Spruck et al. reported that 65% of CIS had a p53 mutation[11]. Therefore, we performed p53 mutation analysis on the various TCC cell lines to test whether CIS-formation was correlated with the presence or absence of p53 mutations in these cell lines (Figure 3). All cell lines contain one or more p53 mutations in exon 5 – 8, which is in concordance with the observed p53-overexpression in the CIS-lesions.

Figure 3: PCR-SSCP tp53 exon 5 of 1207.
In exon 5 of the tp53 gene an aberrant SSCP pattern (arrows) was seen in the 1207 DNA.
Lane 1 DNA from cell line 1207, lane 2 normal control DNA.

Growth and extent of 1207 derived CIS in the bladder

To further define our in vivo model of 1207 derived CIS in the bladder we studied its extent during six months after the inoculation of the 1207 cells (Table 2). CIS of the urinary

bladder was observed in 97% of the cases. The confinement to the urothelium of all these CIS lesions was shown by visualization of the basement membrane by collagen type IV antibody. Only in two mice, besides a number of areas with CIS, a separate invasive bladder tumor was detected at 1 months and at 6 months after intraluminal inoculation.

Table 2. Growth of 1207 Derived CIS in the Mouse Bladder

	14 days	1 month	2 months	5 months	6 months
Number of mice	5	10	10	5	5
TCC/mouse	5/5	10/10	8/10	4/5	4/5
CIS vs. Invasive	5 CIS / 0 Inv.	9 CIS / 1 Inv.	8 CIS / 0 Inv.	4 CIS / 0 Inv.	4 CIS / 1 Inv.
PN[1]/Mice	2/5	7/10	4/10	ND[5]	ND
LI[2] in CIS (mean ± SEM)	18.4 ± 6.7	13.8 ± 5.2	19.3 ± 5.4	18.6 ± 6.2	29.1 ± 6.3
AI[3] in CIS (mean ±SEM)	1.0 ± 1.4	2.5 ± 0.6	3.7 ± 1.9	ND	ND
BMC[4]	0.9	1.0	3.2	22.6	44.7

1) PN = pyelo-nefritis; 2) LI = labeling index; 3) AI = apoptotic index, 4) BMC = % basement membrane covered by CIS[4], 5) ND = not determined.

Probably as a result of the procedure, a high percentage of pyelo-nefritis was detected in the mice. In contrast to the first experiment no tumors were found in other parts of the urinary tract.

Subsequently, we determined the dynamics of the onset of CIS. This was achieved by following the clusters of 1207 cells within the urothelium during the early stages of CIS-formation (Figure 4). The number of aggregates of intraepithelial confined tumor cells increased with time. Beyond two months, large areas of the urothelium are gradually replaced by CIS (Table 2; BMC), reaching an average of 44% at 6 months. The growth potential of the CIS lesions, was further substantiated by the high proliferative activity and low apoptotic index in CIS throughout the whole experiment (Table 2).

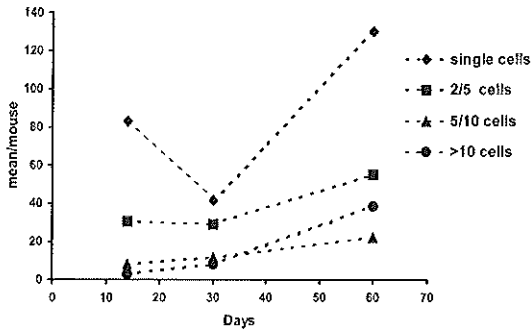

Figure 4: Growth-dynamics (single cells; and groups of 2/5, 5/10, > 10 cells) of 1207-derived CIS at early time-points. Note the steadily increase in larger clusters of 1207 cells in time.

Strikingly, the number of single tumor cells within the urothelium was high at 14 days, fell at 1 month and subsequently rises again at 2 months. Stepwise sectioning and staining with RCK108 of bladders obtained at two months after inoculation revealed that most of these single tumor cells and small clusters of tumor cells could be traced back to a nearby located area of CIS. This suggests that these single cells can be regarded as the pagetoid spread of a nearby CIS.

Discussion

Here we describe the development of a new in vivo model of CIS of the urinary bladder. We demonstrated that intraluminal injection of established human TCC cell lines in denuded mouse bladders consistently resulted in bladder implants. Cell line 1207 generates isolated CIS in the urinary bladder in a high frequency, while complications like invasive carcinoma and metastatic disease were very limited. This makes 1207 the most suitable human TCC cell line to study CIS in vivo.

The growth of the 1207 cell line after inoculation in the urinary bladder was monitored for six months. The extent of the CIS was steadily increasing (Table 2 & Fig. 4). During the six months period the areas with CIS were highly viable as reflected by their high proliferative activity and their low apoptotic index. Invasion was rarely noted in these experiments (Table 2). Strikingly, in a previous experiment, where 1207 cells were injected into the submucosa of the bladder, invasive bladder tumors were formed within 2 months, with an ulcerated surface (Chapter 5). This latter indicates that these same 1207 tumor cells have the ability to degrade the basement membrane. Inoculation of 1207 cells on partially stripped urothelium apparently prevents the invasiveness of 1207 cells. This could suggest that the stromal microenvironment promotes the invasion of 1207 cells, whereas that of the urothelium can prevent the invasion of 1207 cells.

An interesting feature of CIS is the existence of individual or small groups of transformed cells in the normal urothelium adjacent to CIS. This pagetoid type of infiltration is recognized in a considerable proportion (± 10%) of CIS of the human bladder[20]. By mapping studies we found out that a substantial part of the detected single tumor cells at 2 months could be regarded as the pagetoid spread of a nearby located CIS. The ability for pagetoid intraepithelial spreading of 1207 cells is in accordance with the results of our in vitro cocultivation model, in which 1207 cells infiltrate the surrounding normal urothelium as single cells[21].

It is generally accepted that CIS can be regarded as the precursor lesion for invasive bladder carcinoma[6-12]. However, the results from our in vivo model showed that inoculation of otherwise highly invasive TCC cell lines could give rise to the formation of CIS. This suggests that CIS should not only be regarded as a starting point for the progression to invasive carcinoma, but it can also be the result of an alternative mechanism of tumor spread

of high grade invasive carcinoma.

Furthermore, a proportion of the detected CIS consisted of isolated large intraepithelial lesions in the bladder, which were covered by large umbrella cells. According to the WHO grading system these lesions which do not replace the full thickness of the urothelium are called dysplasia, instead of carcinoma in situ[22]. However, the fact that in our model 1207 tumor cells, originally obtained from a highly invasive tumor, gave rise to such lesions suggests that dysplastic lesions can harbor malignant tumor cells and as such they carry a substantial risk of development into an overt carcinoma. This implies that pathologist should be aware of this risk whenever they come across a dysplastic lesion in the bladder.

Previously, other animal models for the generation of CIS of the bladder were described. Treatment of laboratory animals with carcinogenic agents like dibutylnitrosamine (DBN), N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN), N-methyl-N'-nitrosourea (MNU) N-(4-(5-nitro-2-furyl)-thiazolyl formamide (FANFT) and Bracken Fern can induce CIS of the bladder[23-26]. Major drawbacks of these procedures, like the high incidence of papillary and invasive tumors, a long lag time, toxicity to the animal, possible carcinogenicity to other organs, and the often-observed predominance of squamous differentiation, make them less attractive as a model of CIS. Earlier reports on the xenografting of established bladder carcinoma cell lines in bladders of immunodeficient hosts showed low formation of CIS[27-30]. In these models a high incidence of papillary or invasive bladder tumors is reported. Furthermore, because the inoculation of bladder tumor cells in intact bladders is largely prevented by an intact bladder surface, most of these studies used chemical agents or local trauma to promote tumor take[15,31]. However, a major limitation of the latter traumatizing procedures is the chance of deep penetration of the bladder wall thereby facilitating tumor invasion[27]. Obviously, in our model, overstretching of the bladder wall did not result in rupture of the basement membrane and its underlying tissues. This was shown by the preservation of collagen type IV in areas with total loss of the urothelium (Figure 1B). Apparently, this resulted into a high frequency of CIS. In earlier experiments we have shown that intra-muscular inoculation of human TCC, including 1207 led to invasive growth (Chapter 5). Therefore it can be argued that preservation of the basement membrane by employment of a mild procedure to denude the urothelial mucosa may have led to the observed low occurrence of invasive tumors after inoculation of 1207 cells.

In conclusion, the 1207-derived CIS of the bladder is a new, promising and

reproducible model for the study of the behavior of CIS. Since the 1207-derived bladder tumors seem to persist as intraepithelial tumors for a long period (up to six months) it makes them a highly suitable model for the development and testing of intravesical chemo-, immuno- or gene-therapy.

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Chapter 7

GENERAL DISCUSSION

The major problem in the management of superficial bladder carcinoma is the high frequency of recurrences after local resection of the primary tumor. Hypothetically, reseeding and/or (subsequent) intraepithelial expansion (IEE), are two obvious mechanisms that could explain the high recurrence rate of bladder tumors. To study both mechanisms relevant model systems are needed. In this thesis we described the development of useful *in vitro* and *in vivo* model systems of IEE. Subsequently, the described model systems were used in an attempt to identify factors that influence IEE.

In vitro intraepithelial expansion model

In our *in vitro* model, IEE can be considered as the outcome of the balance between the expansion and proliferation of the neoplastic cells and the potential of the surrounding normal urothelium to regenerate. This implies that besides the intrinsic characteristics of the tumor cells, the host environment also influences IEE (Figure 1). In chapter 2, we have shown that we can shift the balance towards the normal urothelium by specific stimulation of its regenerative capacity by growth factors.

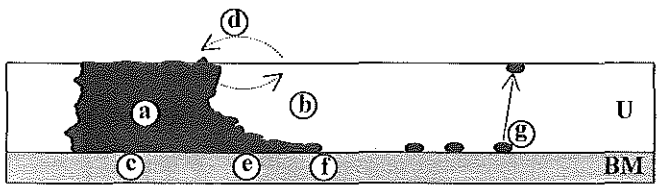

Figure 1: Control of IEE/IEN. Established and suggested mediators (a t/m g; see text) of IEE/IEN are depicted. (U= urothelium; BM= Basement membrane; black= IEN).

E-cadherin-mediated cohesiveness is an important intrinsic characteristic (Fig. 1. a), which determines the IEE-capacity of TCC cells (Chapter 4). However, E-cadherin is not the sole mediator of IEE, since the E-cadherin positive cell line JON hardly showed any IEE. This means that also other, yet unknown factors participate in IEE. For instance integrins-ECM interactions will be involved in the reseeding of tumor cells on the basement membrane (Fig. 1 c) and will probably also be involved in the lateral spread (Fig. 1 e) along the basement membrane. The importance of the latter is corroborated by Harabayashi *et al.*, who noted in their *in vitro* model that a reduction of integrin β4-expression in tumor cells as well as an enhanced migration on laminin are involved in IEE (Harabayashi *et al.*, 1999). Recently, we investigated the involvement of ECM-proteins in the lateral spread of intraepithelial bladder tumors. We

compared the IEE capacity of the TCC cell lines with their expansion on collagen type IV-coated membranes in the absence surrounding urothelium (Table 1; *unpublished results.*).

Table 1: Comparison of IEE (with surrounding urothelium) and expansion on Collagen type IV in the absence of surrounding urothelium.

	IEE	Expansion on Coll. type IV
SD	++	++
T24	-	++
RT112	+	+/-
J82	-	+/-
1207	+	+/-
Jon	-	+/-

Strikingly, we observed that the expansion on collagen type IV not always correlated with the IEE. For instance, the observed IEE of RT112 was proportionally stronger than its expansion on collagen type IV coated membranes in the absence of surrounding urothelium. This suggests that the cocultivation of RT112 with normal urothelial cells facilitate its IEE. We indirectly examined whether excretion of a paracrine substance or deposition of a specific component on the culture substratum stimulates IEE of RT112 cells. We noted that culturing on mouse urothelium deposited culture substratum but not mouse urothelium conditioned medium (Figure 2) stimulated the expansion of RT112 cells in the absence of surrounding urothelium.

Mouse urothelium deposited substratum consists, besides the already coated human collagen type IV, of at least fibronectin, mouse collagen type IV, and laminin (*unpublished results*). By further studies we could demonstrate that fibronectin depositions are probably the most important factor, which contributed to the enhanced expansion of RT112 (*unpublished results*). These results gave further proof of the involvement of integrins-ECM interactions in IEE (Fig. 1 e).

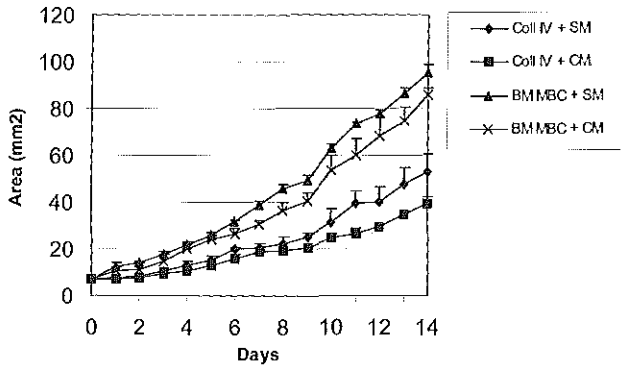

Figure 2: Influence of mouse urothelium conditioned medium (CM) or basement membrane deposited by mouse bladder cultures (BM MBC) on the expansion of RT112 cultures in the absence of surrounding normal urothelium. (Coll IV= collagen IV; SM= standard medium).

Cross-sections of the cocultivations revealed that, like the *in vivo* situation, our TCC cells expand in IEE by undermining (Fig. 1 f) the normal urothelium (Chapter 2). Ultra-structural analysis showed that *in vitro* the normal mouse urothelium is anchored to the basement membrane by hemidesmosomes. Obviously, tumor cells have to disrupt these hemidesmosomes before they undermine the adjacent urothelium (Fig. 1 f). The question remains, how can this be accomplished by the tumor cells? Is mechanical disruption sufficient or is targeted disruption by specialized "invadopodia-like structures" necessary? These invadopodia are cell membrane protrusions which exhibit a coordinated expression of integrins/receptors for proteolytic enzymes and specific proteolytic enzymes, and they are normally involved in disruption of ECM proteins (Kelly *et al.*, 1994; Sato *et al.*, 1994; Brooks *et al.*, 1996).

The production of paracrine substances (peptide growth factors, cytokines, etc.) by the normal urothelium could influence the behavior of intraepithelial tumor cells and vice versa (Fig. 1 d). The importance of the host environment (Fig. 1 b,d) on IEE was suggested by several authors (Orozco *et al.*, 1994; Rebel *et al.*, 1995; Chapter 2). Modulation of the surrounding normal urothelium by growth factors or ECM-proteins resulted in an altered IEE of tumor cells (Rebel *et al.*, 1995; Chapter 2). Orozco *et al.* suggested that, based on the discrepancy in biological behavior of CIS, some patients possess yet unidentified resistance factors for the progression to invasive disease (Orozco *et al.*, 1994). These patients have CIS-lesions, which

exclusively grow in an intraepithelial environment and which usually do not even invade when the adjacent urothelium is heavily damaged (Orozco *et al.*, Murphy *et al.*, 1994).

Recently, Javaherian *et al.* showed that surrounding normal keratinocytes could suppress the intraepithelial growth of malignant keratinocytes (Javaherian *et al.*, 1998). They proposed a mechanism wherein cell contact with normal epithelial cells induces cell cycle arrest and terminal differentiation of tumor cells. Similarly, one could wonder, what will be the fate of single T24 cells (Fig. 1 g) that infiltrated the normal surrounding urothelium? Could direct contact between tumor cells and normal cells prevent the further clonal outgrowth of tumor cells? We studied the expression of cell cycle and apoptotic markers in single T24 cells. Dispersed T24 cells still have a high proliferative activity (MIB-1, BrdU), and hardly show expression of p27 (marker for quiescent cells) (*unpublished results*). These preliminary data suggest that the normal urothelium had no direct effect on the growth of dispersed T24 cells. However, in our *in vivo* model of IEN, single T24 cells often had morphological signs of apoptosis and were discarded from the urothelium by shedding. The latter suggests that shedding of single tumor cells could indeed affect the ability of T24 for IEE (Fig. 1 g).

In vivo model of intraepithelial neoplasia

Inoculation of 1207 cells in partially denuded bladders resulted reproducibly in the frequent formation of IEN. Only in isolated cases, invasive carcinomas of 1207 cells were found, which were probably due to the disruption of the basement membrane during the overstretching of the bladders in our initial experiments (Chapter 6; experiment 1). Long term follow-up showed that in our model no progression from 1207 derived-IEN to invasive carcinomas occurred (Chapter 6). In contrast, inoculation of 1207 in the submucosa of the bladder gave rise to invasive bladder tumors (Chapter 5). There are a few possibilities to explain this difference in behavior of 1207 cells in the two *in vivo* assays. First, in the case of our *in vivo* model of IEN, cells could be selected that gave only rise to IEN. Second, possibly a longer period of time has to pass before the progression to invasive disease eventually will occur. This is however highly doubtful, because even after a follow-up period of 1 year (*unpublished results*) there are still no signs of progression. Third, the specific microenvironment could either stimulate (submucosa) or block (urothelium) the invasive behavior of 1207 bladder tumor cells. The latter would give further evidence for the importance of the host environment in IEE (Fig. 1 b), provided that the

intraepithelial environment indeed promotes IEN-behavior of the 1207 cells.

Future research

Many of the conclusions drawn in this thesis (Chapter 2 & 4) are based on our xenogenic cocultivation model. We are well aware that we can not exclude that some of our findings could be due to differences between the two species. To address this problem, future research should focus on the development of an allogenic cocultivation model. Problems concerning the availability of human biomaterial and the specific labeling of tumor cells have to be solved before this approach can be successful.

To study the role of integrins-ECM interactions in IEE, initial studies should focus on the flowcytometric analysis of integrin-expression and study the expansion of the various TCC cell lines on mouse urothelium deposited culture substratum. Investigations, which compare the exposure of the cocultivations to various ECM-components, will probably not resolve the issue, because exposure to ECM-proteins would affect both tumor cell behavior and composition of the normal urothelium. Correlation studies between the integrin expression and IEE behavior of tumor cells hopefully will give suggestions of specific integrins that are involved in IEE. Next, transfection of TCC cell lines with these specific integrins or the creation of knockouts, and the subsequent testing of these modified cells in our cocultivation assay could further substantiate the role of integrins-ECM interactions in IEE.

We have conducted initial studies on the fate of single cells (T24, etc.). Still more accurate evaluations of the expression of apoptotic and proliferation markers in these dispersed cells are needed. Furthermore, fluorescent labeling of TCC cells and the subsequent *ex vivo* study, using confocal microscopy, could reveal whether shedding is involved in the elimination of these cells.

Confocal microscopy can also be helpful in the study of the presumed targeted expression of combinations of integrins and proteolytic enzymes in "invadopodia-like structures" at the front of undermining bladder tumor cells (Fig. 1 f).

The described *in vivo* model can be used to design therapies that prevent/block the growth of intraepithelial neoplasia and its progression to invasive bladder carcinomas. Furthermore, the results of our *in vitro* IEE assay can now be extrapolated to the *in vivo* IEN

model. For instance, T24 cells stably transfected with E-cadherin or TCC cells with modified integrin expression can be studied in our *in vivo* model and this could demonstrate whether these molecules stimulate the formation of IEN *in vivo*.

Finally, if selection of 1207 cells would take place in our *in vivo* model of IEN, it would also be worthwhile to characterize these cells and compare them with the native 1207 cell population. This could give us further insight into the intrinsic features of tumor cells that contribute to IEN.

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Chapter 8

SUMMARY/SAMENVATTING

Summary

Bladder cancer is the fifth most common malignancy in males in the western society. At initial presentation, ± 65% of the patients has superficial transitional cell carcinomas. The remaining patients have invasive bladder tumors. A big problem in the management of superficial bladder cancer is the high frequency of tumor recurrences after local resection. Based on the presumed monoclonality of these recurrences, the two most obvious mechanisms to explain these recurrences are: 1) Intraluminal shedding with subsequent attachment of tumor cells to traumatized or intact areas in the urothelium; 2) Lateral (intraepithelial) migration of tumor cells into the normal surrounding urothelium. This thesis describes the development of two model systems, which were used to study the role of the above mechanisms in bladder tumor recurrences.

In chapter 2, we describe a new cocultivation model for the study of lateral intraepithelial expansion (IEE). In this model, human bladder tumor cells were seeded on top of traumatized confluent organotypic mouse cultures. These tumor cells preferentially attached to the traumatized areas in the cultures. After implantation, tumor expansion at the expense of the normal surrounding urothelium could be accurately monitored. The implantation and subsequent IEE of bladder carcinoma cell lines, SD and T24, were studied. SD cells expanded into the normal urothelium as a fast growing, sharply demarcated tumor. In comparison, T24 cells infiltrated the normal urothelium as single cells and displayed a slow, but gradual expansion. Next, we studied whether stimulation of the regenerative capacity of the normal urothelium could alter the implantation and IEE of SD or T24 cells. Addition of EGF (epidermal growth factor) stimulated the proliferation of the normal urothelium, and reduced the implantation and growth of T24 cells considerably. The implantation of SD cells was also reduced by addition of EGF, but EGF could not prevent the subsequent expansion of the SD cells. Furthermore, we showed that EGF had no effect on the proliferation or migration of T24 or SD cells. These results suggested that modulation of the host environment (stimulation of regenerative capacity of the normal urothelium by EGF) could reduce implantation to wounded urothelium, and in some instances could alter the IEE of tumor cells.

The effect of EGF on IEE stimulated us to study the role of the EGF-EGFR family during urothelial regeneration in more detail (chapter 3). EGF, TGFα, and to a lesser extent amphiregulin stimulated wound regeneration of normal human urothelium *in vitro*. Since these growth factor treatments had no general effect on urothelial proliferation during wound healing, the enhanced regeneration could be primarily attributed to an increase in cellular migration.
C-erbB2 plays an important coordinatory role in the function of the EGFR family. Therefore, the role of c-erbB2 in urothelial regeneration was further investigated with the use of antisense DNA specific for c-erbB2. Urothelial reepithelialization could be delayed up to 50% by antisense c-erbB2, but not by mismatched or sense c-erbB2-oligonucleotides. The proliferative capacity of the urothelial cultures was not altered after treatment with any of the oligonucleotides. This suggests that c-erbB2 is a regulator of migration during urothelial wound healing.

Using our *in vitro* cocultivation assay, we compared the IEE of a panel of six bladder carcinoma cell lines (chapter 4). In this way, we hoped to identify factors intrinsic to tumor cells that determine IEE. IEE was most pronounced in three (SD, RT112, and 1207) of four E-cadherin positive cell lines. In contrast, the two E-cadherin negative cell lines (T24 and J82) were hardly able to expand into the normal urothelium. This suggests that E-cadherin is an important mediator of IEE. To further test this assumption, we transfected T24 cells with full-length mouse E-cadherin cDNA. Only T24 clones with a high, functional expression of E-cadherin displayed an enhanced IEE expansion rate. Transfection did not alter their proliferative capacity, or their pattern and level of integrin expression, or their ability to expand on collagen type IV coated membranes in the absences of urothelium. These data suggest that E-cadherin-mediated cohesiveness is an important determinant of IEE of bladder carcinoma cells.

In general, intraepithelial neoplasia (IEN) is considered as an important precursor lesion of invasive bladder carcinoma. However, not all observed IEN will ultimately lead to invasive disease. Therefore, in chapter 5, we investigated the invasive capacity of the previously used bladder carcinoma cell lines. This has further characterized our panel of cell lines, and hopefully will contribute to the future understanding of the paradoxical behavior of IEN, like CIS. In other organ systems it was demonstrated that the microenvironment has an important influence on the behavior of tumor cells. To investigate the importance of the

microenvironment in bladder cancer invasion, bladder carcinoma cell lines were tested in both *in vitro* and *in vivo* invasion assays. *In vitro* invasion into embryonic chicken heart fragments was inversely correlated with their E-cadherin expression. However, the results of this *in vitro* assay could not predict *in vivo* invasiveness. The *in vitro* non-invasive cell line SD formed highly invasive tumors *in vivo*. This could be ascribed to the observed heterogeneous expression of E-cadherin in these SD tumors. In addition, we also observed site-specific tumor take for 1207 and T24 bladder carcinoma cells. These results show that the urinary bladder microenvironment indeed plays an important role in tumor growth (1207 and T24) and in the induction of the invasive phenotype (SD).

Taken together, the results described in chapter 4 and 5 suggest that E-cadherin plays a paradoxical role in bladder tumorigenesis. On the one hand, E-cadherin promotes the expansion of intraepithelial neoplasia; on the other hand, its loss correlates with invasive behavior of bladder tumors.

To extrapolate our *in vitro* findings to the more complex situation of humans, relevant *in vivo* models are needed. To establish an *in vivo* model of IEN, bladder carcinoma cells were intraluminally injected in partially denuded mouse bladders (chapter 6). The denuded bladders were rapidly reepithelialized with mouse urothelial cells and the injected tumor cells. Four TCC cell lines were compared for their ability to form IEN. All four cell lines showed formation of IEN. However, there were considerable differences in the frequency of IEN formation. Furthermore, invasiveness and development of tumors elsewhere in the urinary tract were also observed. Cell line 1207 was identified as the most promising and useful model of IEN, because this cell line revealed the highest frequency of IEN in the bladder, and long-term experiments were less likely to be hampered by invasive carcinoma or metastatic spread. In the following time-course experiment, we studied the growth characteristics of these 1207-derived IEN during six months. In this experiment, 1207 again displayed a frequent formation of IEN, and invasion was only found in isolated cases. During the six months period, 1207-derived IEN showed signs of gradual intraepithelial expansion. The growth of these 1207-derived IEN was based on a high proliferation rate and a low apoptotic rate. Intraluminal injection of 1207 cells in partially denuded bladder results in a highly reproducible model for the study of the behavior of IEN. In addition, our model also suggests

that formation of IEN can be the result of an alternative mechanism of tumor spread of high-grade invasive bladder carcinoma.

Samenvatting

Blaaskanker is in de westerse samenleving de vijfde meest voorkomende maligniteit. Van de patiënten met overgangsepitheel carcinoom van de urineblaas heeft 65% superficiële tumoren. De overige patiënten hebben invasief blaascarcinoom. Een groot probleem in de behandeling van de superficiële tumoren is het hoge recidief percentage en de kans op progressie na locale resectie van de primaire tumor. Uitgaande van de veronderstelde monoclonaliteit van blaastumoren, zijn er twee mechanismen mogelijk, nl.: 1) loslaten van blaastumorcellen van de primaire tumor, gevolgd door hechting van blaastumorcellen op de (verwondde) blaaswand; 2) Laterale (intra-epitheliale) migratie van tumorcellen in het normale urotheel. Dit proefschrift beschrijft de ontwikkeling van twee model systemen, die gebruikt werden om de rol van beide mechanismen bij de vorming van blaaskanker recidieven te bestuderen.

In hoofdstuk 2 wordt een nieuw cocultivatie model beschreven. In dit model, worden suspensies van humane blaastumorcellen uitgezaaid op een beschadigde culture van urotheel van de muizenblaas. Na hechting van de tumorcellen in de beschadigde gebieden, kan de expansie van deze tumorcellen ten opzichte van het omringende, normale urotheel worden onderzocht.

De hechting en de daaropvolgende intra-epitheliale expansie van de twee humane blaaskanker cellijnen SD en T24 werden in dit model onderzocht. De SD tumorcellen expandeerden als een snel groeiend, scherp begrensd veld zonder infiltratie van het normale urotheel. Daarentegen expandeerden T24 tumorcellen langzaam, waarbij individuele T24 tumorcellen het normale urotheel infiltreerden. Vervolgens werd bestudeerd of stimulatie van de regeneratieve capaciteit van het normaal urotheel, de hechting en intra-epitheliale expansie van T24 of SD cellen kon beïnvloeden. Epidermale groei factor (EGF) stimuleerde de proliferatie van het normale urotheel en zorgde voor een gereduceerde hechting en groei van T24 cellen. De hechting van SD cellen werd eveneens gereduceerd door toevoeging van EGF. Er vond echter geen remming plaats van de expansieve groei van de SD tumorcellen na EGF-

toevoeging. Het veranderd gedrag van beide tumorcellijnen kon niet toegeschreven worden aan een door EGF-geïnduceerde verandering van de proliferatieve of migratoire activiteit van beide tumorcellijnen. Dit suggereert dat stimulatie van de regeneratieve capaciteit van het normaal urotheel kan leiden tot een verminderde hechting van blaastumorcellen op beschadigd urotheel en evencens in sommige gevallen in een veranderde intra-epitheliale expansie van blaastumorcellen.

Het effect van EGF op de intra-epitheliale expansie stimuleerde ons om de rol van de EGF/EGF-receptor familie tijdens de regeneratie van beschadigd urotheel nader te onderzoeken (hoofdstuk 3). De *in vitro* regeneratie van normaal humaan urotheel werd gestimuleerd door toediening van EGF, TGFα of amphireguline. Dit effect kon voornamelijk toegeschreven worden aan een toegenomen migratie van urotheelcellen, die zorgden voor het sluiten van de beschadiging. C-erbB2 fungeert als een belangrijke coördinator voor de functie van de EGF-receptor familie. Daarom werd de rol van c-erbB2 tijdens de regeneratie verder onderzocht door het gebruik van een antisense-DNA strategie. Regeneratie van het urotheel werd aanzienlijk geremd (± 50%) door toediening van antisense c-erbB2, terwijl negatieve controles geen effect lieten zien. De proliferatieve capaciteit van de beschadigde urotheelkweken werd door geen van deze oligonucleotides veranderd. Dit suggereert dat, tijdens de regeneratie, c-erbB2 vooral een belangrijke regulator van urotheelcel-migratie is.

In hoofdstuk 4 werd de intra-epitheliale expansie van zes verschillende blaaskankercellijnen onderling vergeleken in van het eerder beschreven cocultivatie model. Op deze wijze hoopten we intrinsieke factoren van de tumorcellen te identificeren, die betrokken zijn bij het proces van intra-epitheliale expansie. Bij drie van de vier E-cadherine positieve cellijnen (SD, RT112 en 1207) werd de grootste intra-epitheliale expansie waargenomen. Dit, terwijl de twee E-cadherine negatieve cellijnen, T24 en J82, vrijwel geen intra-epitheliale expansie toonden. Dit zou kunnen beteken dat de aanwezigheid van E-cadherine een belangrijke bijdrage levert aan de intra-epitheliale expansie van blaastumoren. Om deze aanname verder te onderzoeken, werden T24 cellen met het cDNA van E-cadherine getransfecteerd. T24 klonen met verschillende mate van E-cadherine expressie werden in onze cocultivatie assay getest. Alleen T24 klonen met een hoge functionele expressie van E-cadherine hadden een toegenomen intra-epitheliale expansie. Transfectie van T24 cellen met E-cadherine had geen effect op de

proliferatieve activiteit, noch op het expressiepatroon cq. expressieniveau van verschillende integrines, noch op de expansiesnelheid op collageen type IV-gecoate membranen in de afwezigheid van omringend urotheel. Hieruit kan geconcludeerd worden, dat E-cadherine-gemedieerde tumor-cohesie een belangrijke bijdrage levert aan de intra-epitheliale expansie van blaastumoren.

In het algemeen wordt intra-epitheliale neoplasie in de blaas beschouwd als een belangrijke voorloper van invasief blaascarcinoom. Echter niet elke waargenomen intra-epitheliale neoplasie zal zich ontwikkelen tot een invasief blaascarcinoom. Inmiddels is bekend dat de omgeving een belangrijke invloed heeft op het invasieve gedrag van tumorcellen. De invasieve capaciteit van de door ons eerder gebruikte cellijnen werd onderzocht (hoofdstuk 5) zowel in *in vitro* als *in vivo* invasie assays om meer inzicht te krijgen in de factoren, die een rol spelen bij de progressie naar invasief gedrag van intra-epitheliale neoplasie. *In vitro* invasie was sterk gecorreleerd aan de *in vitro* expressie van E-cadherine. De resultaten verkregen met deze *in vitro* invasie assay konden echter het invasieve gedrag *in vivo* niet altijd voorspellen. Bijvoorbeeld, cellijn SD liet in de *in vitro* assay geen invasiviteit zien, terwijl *in vivo* hoogst invasieve tumoren werden gevormd. Dit kan mogelijk verklaard worden door een afname in E-cadherine expressie in deze tumoren. Verder werden er bij andere cellijnen blaas-specifieke tumorgroei en invasiviteit waargenomen (1207 en T24). Bovengenoemde resultaten laten zien dat de omgeving waarin een tumorcel zich bevindt inderdaad een belangrijke rol speelt in de groei (1207 en T24) en invasiviteit (SD) van tumorcellen.

De resultaten uit hoofdstuk 4 en 5 suggereren een paradoxale rol voor E-cadherine in blaaskanker. Enerzijds stimuleert de aanwezigheid van E-cadherine the intra-epitheliale expansie van blaastumorcellen, anderzijds is verlies van E-cadherine expressie gecorreleerd aan invasief gedrag.

De eerder ontwikkelde cocultivatie assay (hoofdstuk 2) is een zeer sterke vereenvoudiging van de complexe situatie, in het geval van blaaskanker bij de mens. Om een beter inzicht te krijgen in het proces van intra-epitheliale expansie zijn daarom relevante diermodellen zeer gewenst. Om die reden werd door ons een diermodel voor intra-epitheliale neoplasie

ontwikkeld (hoofdstuk 6). In dit model worden humane tumorcellen in het lumen van de urineblaas van een muis geïnjecteerd, waarbij eerder een gedeelte van hun urotheel was verwijderd. In deze situatie kan een nieuwe bekleding van de blaas ontstaan, die deels bestaat uit normaal urotheel, deels uit tumorcellen. Vier blaastumorcellijnen werden op deze wijze vergeleken met betrekking tot de vorming van intra-epitheliale neoplasie. De injectie met cellijn 1207 resulteerde het meest frequent in de vorming van intra-epitheliale neoplasie, terwijl invasieve tumoren zelden gevonden werden. Daarom werd besloten om met deze cellijn verder te gaan voor de ontwikkeling van een diermodel. In de daaropvolgende experimenten werd de groei van intra-epitheliale neoplasie in het 1207-model gedurende zes maanden vervolgt. Gedurende deze onderzoeksperiode werd een gestage groei waargenomen van de 1207 tumoren zonder invasieve groei. De toename van deze intra-epitheliale neoplasie was het gevolg van een hoge mate van proliferatie en een lage mate van apoptose. Injectie van 1207 cellen in het lumen van gedeeltelijk beschadigde muizenblazen is een goed reproduceerbaar diermodel, dat bruikbaar is bij de studie naar het gedrag van intra-epitheliale tumoren. Onze resultaten suggereren bovendien, dat hooggradig, invasief blaascarcinoom zich elders in de blaas kan manifesteren als intra-epitheliale neoplasie.

Curriculum Vitae

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List of Publications

De Boer WI, Vermey, M, Diez de Medina SG, Bindels, E, Radvanyi, F, Van Der Kwast, T, and Chopin, D. Functions of fibroblast and transforming growth factors in primary organoid-like cultures of normal human urothelium. Lab Invest, 75: 147-156, 1996.

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Bindels, EMJ, Van Der Kwast, ThH, Chopin, DK, De Boer, WI. Functions of epidermal growth factor-like growth factors during human urothelial recpithelialization *in vitro* and the role of ErbB2. Submitted.

Bindels, EMJ, Vermey, M, De Both, NJ, Van der Kwast, ThH. Influence of the microenvironment on invasiveness of human bladder carcinoma cell lines. Submitted.

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