TUMOUR CELL EXPANSION IN BLADDER EPITHELIUM

EXPANSIE VAN TUMOR CELLEN IN BLAAS EPITHEEL

PROEFSCHRIFT

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AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
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DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 19 APRIL 1995 OM 15.45 UUR

DOOR
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GEBOREN TE BUSSUM

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Dit proefschrift werd bewerkt binnen de vakgroep Pathologie van de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam. Het onderzoek en deze uitgave zijn tot stand gekomen met financiële steun van de Nederlandse Kanker bestrijding-Koningin Wilhelmina Fonds.

aan Peter

CONTENTS

LIST OF ABBREVIATIONS

aFGF	acidic fibroblast growth factor
BCG	Bacillus Galmette Guerin
bFGF	basic fibroblast growth factor
CIS	carcinoma in situ
Col I	collagen type I
Col IV	collagen type IV
ECM	exracellular matrix
EGF	epidermal growth factor
FCS	fetal calf serum
FN	fibronectin
IGF	insulin like growth factor
Lam	laminin
PBS	phospate buffered saline
PDGF	platelet derived growth factor
pp125 ^{fak}	pp125 focal adhesion kinase
TCC	transitional cell carcinomas
TGF	transforming growth factor
TUR	trans-urethral resection

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Bladder cancer is common in western society. The major problem of patients with superficial bladder cancer (Ta/T1) is the high recurrence rate and multifocality of these tumours. In 70% of the patients superficial bladder cancer recurs after local resection of the tumour within 15 years. The tumour recurrences are probably caused by tumour cells that were not removed by the therapy. These residual tumour cells apparently can form secondary tumours at multiple sites in the bladder mucosa. In this respect bladder cancer is different from other carcinomas. Factors involved in the normal physiology of the bladder epithelium may also attribute to the expansion of tumour cells and the recurrence of bladder carcinomas. In order to provide better understanding of the process of bladder cancer recurrence the anatomy and histology of the urinary bladder and the carcinogenesis of bladder carcinoma are described in paragraphs 1.1 and 1.2. In paragraph 1.3 the development of tumour recurrences is described and a hypothesis is developed regarding the role of extracellular matrix components, growth factors and adhesion molecules in the development of recurrences, either by affecting the normal bladder epithelium or by affecting the bladder carcinoma cells themselves (paragraph 1.4).

1.1 THE BLADDER

Structure of the bladder

The urine from the kidneys is directed to the urinary bladder via the ureters. The urinary bladder is a hollow organ that serves as a temporary reservoir for urine that can be voluntarily voided at an appropriate time. The bladder wall and ureter consist of three distinct layers: the mucosa, the lamina propria and a smooth muscle layer. The mucosa bordering the lumen, rests on a thin lamina propria composed of loose connective tissue. This connective tissue is in contact with a thick smooth muscle layer (Hicks, 1975).

The mucosa of the bladder and ureters consists of transitional epithelium, also called urothelium. The urothelium of the human bladder consists of 6 to 7 cell layers while the murine urothelium consists of 3 cell layers (Jost, 1989). In all species, the urothelium shows a similar maturation pattern. At the basal side of the urothelium, separated from the lamina propria by a basement membrane, are the basal cells. The basal cells have the morphology

of small undifferentiated cells. These cells have the ability to proliferate rapidly after injury. The top layer of the urothelium (lining the lumen) consists of large terminally differentiated cells, the umbrella or superficial cells. Between these layers are the intermediate cells which are larger than the basal cells and smaller than the umbrella cells (Fig 1). The umbrella cells are the progeny of the basal cells. During maturation of basal cells to umbrella cells a gradual upward migration of maturing epithelial cells occurs.

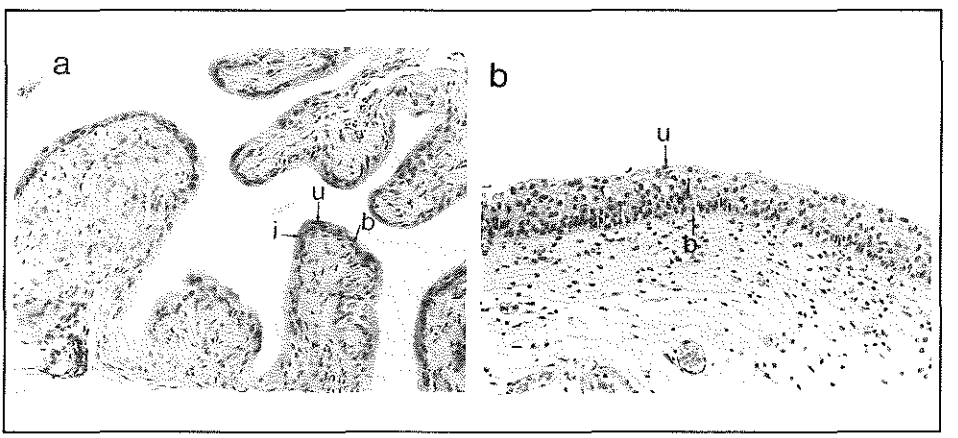

Figure 1-Overview of the bladder b are the basal cells, i the intermediate cells and u the umbrella cells a) a murine bladder b) a human bladder

The surface of the umbrella cells is covered by glycosaminoglycans, proteoglycans and glycoproteins to prevent adhesion of bacteria and proteins in the urine to the urothelium. These glycoproteins protect the urothelium and the underlying layers from bacterial invasion (Parsons et al., 1975, 1977, 1985).

The cell turnover in unstimulated mammalian and murine urothelium is very low (Levi et al., 1969, Mackillop et al., 1985). The cell cycle of a basal cell takes $\pm$ 60 hours. Mitotic figures are mostly found in the basal cell layer but its mitotic index is very low $\leq 1\%$ (Jost, 1989). An occasional proliferating cell can be found in more superficial cell layers (Messier et al., 1960). This suggests that even highly differentiated cells (the umbrella cells) retain the ability to proliferate.

Urothelial regeneration

The regenerative potential of the urothelium after denudation of the bladder mucosa has been studied in several species, including man and mouse and is known to be very high (Liang et al., 1963, Hansen et al., 1969, Lund, 1969, Wishnow et al., 1989). This is in contrast with the low turnover under normal circumstances. After stripping the urothelium from the whole mouse bladder, the bladder is again covered with urothelium within 48 hours. In a patient study Liang et al. (1962) reported that after removal of 75% of the bladder urothelium, the bladder wall is re-epithelialized within a month. Regenerating urothelium may have originated from the free edges of the remaining urothelium, from the vesical neck or from the (prostatic) urethra. Several mechanisms may account for rapid re-epithelialization of the bladder surface:

a) an increase in proliferation of the remaining urothelial cells. In regenerative urothelium the umbrella cells are also able to proliferate (Levi et al., 1969).

b) migration out of the urethra and ureters (Connolly et al., 1971, Wishnow et al., 1989).

c) extension of the surfaces of remaining cells by flattening of the cell body (Hicks, 1975).

Which of these mechanism is actually involved and how this is regulated is not completely resolved.

1.2 BLADDER CANCER

Epidemiology and etiology

The risk for developing bladder cancer increases with age, with a peak incidence between 60 and 70 years. Bladder cancer affects men three times more often than women. It is the fifth most common cancer in western society, with an annual incidence of 20 cases per 100,000 in industrialized countries (Raghavan et al., 1990). In less industrialized countries in Asia and Africa the incidence of carcinoma of the bladder is lower except for regions where *Schistosomiasis* is endemic (Gelfand et al., 1967). This parasite gives rise to a long term chronic irritation and inflammation. As a consequence the transitional epithelium is replaced by squamous epithelium. This metaplastic squamous epithelium can progress to squamous cell carcinoma due to the persistence of the inflammation.

Epidemiological studies have implicated that cigarette smoking and the development of bladder cancer are related (Hoover et al., 1971, Armstrong et al., 1974, Soloway, 1992). The risk for developing bladder cancer among smokers is two to three times higher than among non-smokers. A proven relation between occupation and bladder carcinoma also exists (Hoover et al., 1973, Silverman et al., 1989). An increased risk for bladder cancer was found for painters, truck drivers, hair dressers and drill press operatives. Owing to their profession these people are exposed to different carcinogens like the aromatic amines (Lower, 1982).

Pathology

Bladder carcinomas mostly develop in the transitional epithelium of the bladder and largely consist of transitional epithelial cells, without significant squamous cell differentiation. These tumours are called transitional cell carcinomas (TCC). In industrialized countries squamous cell carcinomas of the bladder occur less frequently. They are not discussed here because they are beyond the scope of this thesis.

TCC can be divided in:

1) Papillary urothelial tumours of the bladder. Regardless of the grade of the papillary tumours their gross appearance is a sea anemone-like structure (Fig 2a). The central tissue stalk is the connection to the bladder wall, carrying the blood supply for the tumour. These tumours are graded as follows:

* Grade I tumours are characterized by papillary structures lined with epithelial cells lacking significant nuclear abnormalities. The number of cell layers of the epithelium is increased ($\geq$ 8) compared to normal urothelium. The cells lining the surface show a differentiation to umbrella cells.

* Grade II tumours are tumours which have characteristics intermediate between grade I and III tumours.

* Grade III tumours are characterized by a more or less disturbed and disorderly epithelium with substantial nuclear abnormalities. In grade III tumours no superficial cells with maturation to umbrella cells are found (Koss, 1975, Robbins et al., 1981).

2) Non-papillary (solid) tumours of the bladder. These tumours are flat, plaque-like tumours which acquire invasive properties. There is a variability of cells with respect to size, appearance of cytoplasm and the degree of anaplasia. Most of the solid tumours are grade III tumours.

Bladder carcinomas are staged according to the depth of invasion into the bladder wall. Stage T_a bladder carcinomas are papillary carcinomas that do not infiltrate into the bladder wall. T_1 tumours are superficially infiltrative into the lamina propria and the submucosa, while T_2 tumours extend into the deep muscle layer.

High grade papillary carcinomas (grade III) and non-papillary carcinomas are usually associated with other urothelial abnormalities such as intra-epithelial neoplasia and hyperplasia. The most common form of intra-epithelial neoplasia is the carcinoma in situ (CIS), another less common form is the pagetoid infiltration. CIS is characterized by epithelium with obvious nuclear abnormalities and a disorderly pattern of growth. The superficial cell layer with maturation to umbrella cells is not seen. Cell size and thickness are quite variable. CIS may represent the intra-epithelial propagation of tumour cells originating from a grade III papillary carcinoma. However, CIS can also occur without an

accompanying infiltrating carcinoma, and can then be regarded as a precursor lesion of the non-papillary infiltrating carcinoma (Koss, 1975, Orozco et al., 1994). The pagetoid type of infiltration is characterised by penetration of individual neoplastic cells, out of the carcinoma, into otherwise morphologically normal stratified epithelium (Ortega et al., 1953, Orozco et al., 1993).

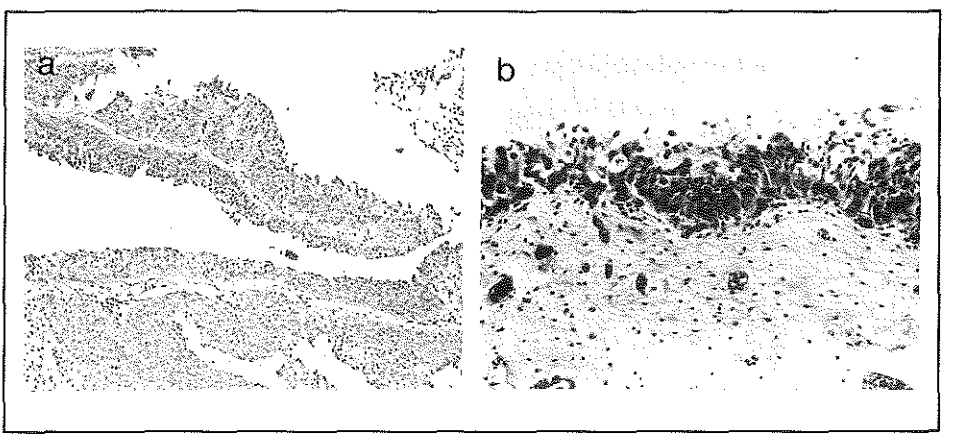

Figure 2- a) frond-like structures of a papillary transitional cell carcinoma b) a carcinoma in situ

DNA changes in bladder cancer

An accumulation of mutations in oncogenes and tumour suppressor genes can change a normal cell into a tumour cell. In bladder cancer mutations in the H-ras oncogene and the cERB-2/neu oncogene have been found. Several, so far mostly unidentified, tumour suppressor genes play a role in the pathogenesis of bladder cancer as judged from the loss of specific chromosomal domains.

Cytogenetic studies have defined numerical and structural changes involving chromosomes 1, 3, 5, 6, 7, 9, 11 and 17. (reviewed by Sandberg et al., 1994). In general, loss of chromosome 9 occurs independent of tumour progression and this is common to all urothelial tumours (Tsai et al., 1990, Hopmann et al., 1991, Cairns et al., 1994, Knowles et al., 1994). Loss of chromosome 9 may represent an early event.

Loss of chromosome 17p frequently occurs in tumours which have invaded the lamina propria or muscle layer but not in low stage tumours (papillary, non-invasive) (Olumi et al., 1990, Habuchi et al., 1992). The p53 gene, situated on the short arm of chromosome 17

codes for the p53 tumour suppressor protein. p53 mutations also occur in papillary tumours invading the lamina propria (Sidransky et al., 1991, Esrig et al., 1993, Cordon-Cardo et al., 1994). Non infiltrating (superficial) papillary tumours do not have mutations in p53 and no loss of chromosome 17 has been observed. The above findings suggest that allelic loss of chromosome 17p and mutations in the p53 gene are late events in bladder carcinogenesis.

On the other hand, Spruck III et al. (1994) found in 65% of CIS specimens mutations in the p53 gene and no detectable loss of chromosome 9. In low grade papillary tumours they found in 34% of the cases loss of chromosome 9 with no p53 mutation. They suggest that there are at least two molecular pathways by which a TCC could develop (Fig 3). Loss of p53 without loss of chromosome 9 can give rise to the development of a carcinoma in situ. In contrast, loss of chromosome 9 without mutations in the p53 gene is associated with the development of low grade papillary tumours.

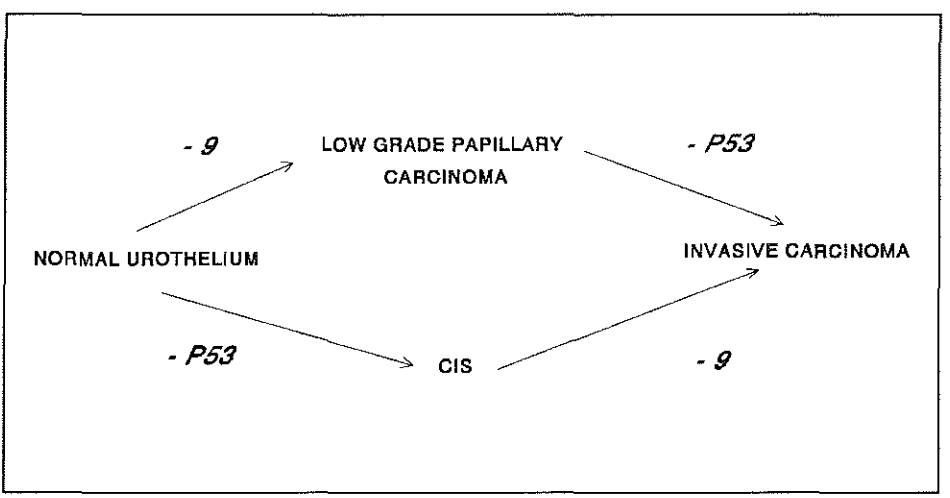

Figure 3-Proposed model for the progression of bladder carcinoma based on allelic loss of chromosome 9 and p53 mutations. (Figure adapted from Spruck III et al., 1994)

Bladder cancer treatment

Superficial bladder tumours (Ta/T1) are treated by trans-urethral resection (TUR). Whereas recurrences of Ta/T1 bladder tumours are frequently observed (70%) only a proportion will progress into tumours with stage T2 (13%) (Kurth et al., 1992). After removal of a superficial bladder tumour the regenerative capacity of the urothelium permits a rapid re-epithelialization. Intravesical installation of, for example, mitomycin C is applied

when CIS is observed. The subsequent inhibition of DNA synthesis preferentially kills the rapidly proliferating neoplastic cells. More often a Bacillus Galmette Guerin (BCG) installation is given to treat CIS and superficial (T_a) papillary tumours. The exact mode of action of BCG is not known. Probably BCG attaches to the superficial cells of the urothelium and an inflammatory process is induced. This therapy is effective in reducing the frequency of tumour recurrences. If the TCC has invaded the deep muscle layer (T2) a total cystectomy or radiotherapy is performed (Macfarlance, 1988).

1.3 BLADDER CANCER RECURRENCES

Recurrent bladder carcinomas: monoclonal or multiclonal

In spite of the therapies applied to control bladder cancer and the fast regenerative capacity of the urothelium, the major problem in patients with superficial bladder cancer is the high recurrence rate. Between 33 and 80% of the bladder tumours recur and up to 42% of TCC pregress in stage. (Hinman, 1956, Heney et al., 1983, Kurth et al., 1992, Levi et al., 1993). These newly formed tumours could be: (1) de novo appearance of a malignancy of independent origin in the bladder (multifocal origin), based on the assumption that most urothelial cells would have been exposed to carcinogens or (2) the progeny of the primary tumour; this would imply that all tumours are of monoclonal origin.

Sidransky et al. (1992) have demonstrated that multiple simultaneously occurring bladder carcinomas in a single cystectomy specimen are of monoclonal origin. They found the same inactivated X chromosome in different bladder tumours of the same patient, while in normal bladder mucosa random patterns of inactivation of the X chromosome were observed. Each of the tumours, within the same patient, also had the same allelic loss of chromosome 9. They concluded that the tumours must have arisen from the uncontrolled spread of a single transformed cell. Similarly, a report from Habuchi et al. (1993) suggested that tumour recurrences in the bladder are due to monoclonal tumour spreading. They detected identical p53 gene mutations in the primary and recurrent tumours. Miyao et al. (1993) also demonstrated that recurrent tumours and metastatic nodules in the individual patient contained identical genetic lesions and were therefore probably of monoclonal origin.

The above findings support the conclusion that multifocal and recurrent tumours are of monoclonal origin, which contrasts with the original hypothesis of a polyclonal origin of recurrent bladder carcinomas.

Pathways that might be involved in the development of recurrences in bladder cancer

The idea that the recurrent tumours are monoclonal leads to the conclusion that the tumour cells from the primary tumour must be able to expand in the urothelium. How this tumour cell expansion process takes place is not known yet. However, because of the high recurrence rate of bladder cancer it is important to know how transformed urothelial cells can replace the normal (regenerating) urothelium. Two major pathways could lead to bladder

carcinoma recurrences:

1) The shedding of tumour cells from the primary tumour in the urine with subsequent attachment to damaged or even intact urothelium and the formation of new carcinomas at distant sites. (Hinman, 1956, Soloway et al., 1980). Tumour cells can indeed implant randomly in the bladder after TUR of the bladder carcinoma. When bladders of rats or mice are injured and tumour cells are inoculated in the bladder, implantation of tumour cells occurs mostly in the traumatized areas of the bladder (See et al., 1989, 1990, 1992, Summerhayes et al., 1988, Pan et al., 1989). In patients with bladder carcinoma often multiple tumours are found at first diagnosis (Lutzeyer et al., 1982); these tumours could not have developed by implantation of tumour cells on traumatized urothelium. A more plausible explanation would be the implantation and colonization on intact urothelium. Soloway et al. (1980) noted tumour cell implantation in 12% of the non-injured mouse bladders, while in 54% of the injured bladders implantation was found. The mechanisms by which the attachment of transformed urothelial cells to the urothelium occur, could either be cell-cell adhesion when the urothelium is intact and/or cell-extracellular matrix component adhesion when the urothelium is traumatized after for example TUR.

2) Outgrowth of transformed urothelial cells from a carcinoma replacing the normal urothelium by intra-epithelial expansion (Mahadevi et al., 1986). Koss et al. (1974) found that (infiltrating) bladder carcinomas are often surrounded by areas with pre-cancerous urothelial lesions, with atypical and hyperplastic urothelium forming a carcinoma in situ or occasionally, displaying a pagetoid form of infiltration. This 'field cancerization' may be the consequence of replacement of normal urothelium by a intra-epithelial expansion of transformed urothelial cells originating from an adjacent tumour. In the mechanism by which the intra-epithelial expansion of transformed urothelial cells occurs, cell migration must be involved.

Strategies towards reducing the recurrence rate of TCC

To decrease the recurrence rate of TCC the balance between the expanding transformed urothelial cells and the normal regenerating urothelium must be influenced. Cell-extra-cellular matrix (ECM) adhesion (implantation of tumour cells on traumatized urothelium), cell-cell adhesion (implantation on intact urothelium) and cell migration (intra-

epithelial expansion of tumour cells) can be influenced by external factors like ECM components, growth factors and cell adhesion molecules. When implantation on (traumatized) urothelium and intra-epithelial expansion in urothelium of transformed urothelial cells could be inhibited, the number of tumour recurrences could be reduced.

1.4 EXTRACELLULAR MATRIX COMPONENTS, GROWTH FACTORS AND CELL ADHESION STRUCTURES

General properties of extracellular matrix components

The extracellular matrix consists of the basal laminae and the interstitial stroma. Basal laminae are 20-200 nm broad deposits of specific proteins that provide support for cells and tissues. They are considered to influence cell morphology, cell adhesion, and tissue invasion. Basal laminae are normally produced by epithelial cells and many mesenchymal cell types. ECM proteins typically found in basement membranes are collagen type IV, laminin, fibronectin, a heparan sulphate proteoglycan and some additional components.

Collagen type IV is an insoluble protein consisting a heterotrimer of different α chains. Laminin is a large glycoprotein with an A, B1 and B2 chain which has a unique cruciform structure. Laminin can bind to collagen type IV. Fibronectin is a large extracellular glycoprotein composed of structurally similar subunits. Fibronectin can also bind to collagen type IV. The above mentioned ECM components can all mediate cell attachment, migration and invasion (Liotta et al., 1986, Timpl, 1989). The ability for cells to adhere to ECM proteins is important for a number of processes including migration.

Different receptors specific for these ECM proteins have been identified. The family of integrins consists of receptors that allow cells to adhere to extracellular matrix components. Integrins are integral membrane heterodimeric glycoproteins composed of noncovalently associated α and ß chains. Each combination of α and ß chains in the integrin heterodimer has a unique ligand specificity (Buck, 1987, Takada et al., 1988, Cassiman, 1989, Albelda et al., 1990).

Signals from the ECM components are transmitted via the integrins to the intracellular machinery and the nucleus. This controls cell migration and spreading. The precise mechanism of signal transduction is not yet known. Clustering of the integrin receptors and cytoskeletal reorganization enhanced the tyrosine phosphorylation of pp125 focal adhesion kinase ($pp125^{FAK}$). How the reorganization of actin filaments in the cytoskeleton activates the $pp125^{FAK}$ and how this $pp125^{FAK}$ induces migration and gene expression is not known yet (Juliano et al., 1993).

Influence of ECM components on migration and attachment

Cells have to attach to the ECM before migration or cell spread can occur. *In vitro* models have shown that different cell types can attach and spread on laminin, fibronectin and on collagen type IV (Papadopoulos et al., 1990, Massia et al., 1993, Ohiji et al., 1993). Migration of different cell types on ECM components occurs for example during wound healing. *In vivo* studies have been performed to investigate which specific ECM components stimulate the regeneration process during wound healing. For wounds in pig epidermis an increased laminin expression around the wound was observed. In intestinal defects migration of the epithelium is stimulated by implantation of matrigel, a mixture of different basement membrane components, onto the lesion (Thompson, 1990, Rigal et al., 1991, Rigal et al., 1992).

In vitro studies have shown that collagen types III and IV stimulate intestinal epithelial migration and that fibronectin induces cornea epithelial migration (Nishida et al., 1983, 1990, Moore et al., 1992). Laminin stimulates neuronal and smooth muscle cell migration (Calof et al., 1991, Clyman et al., 1994). Migration of endothelial cells could be stimulated by laminin, collagen type IV and fibronectin (Kirkpatrick et al., 1990, Hauser et al., 1993). Thus attachment and migration of different cell types can be stimulated by ECM components. However, not all cell types show migration on the same ECM components.

ECM components in the urinary bladder

In the basement membrane of the urothelium laminin, collagen type IV and fibronectin are present. In TCC a correlation was found between loss of basement membrane and degree of tumour invasiveness. In some T_a tumours degradation of the basement membrane was observed already before the tumour became invasive. But even when the tumour is invasive the urothelial tumour cells themselves can produce ECM components. No relation between ECM degradation or production and tumour recurrence was found (Conn et al., 1987, Schapers et al., 1990). Bladder carcinoma cells (for example the human T24 cells) can attach to different ECM components. The rat bladder carcinoma NBT II cells can attach to laminin, fibronectin and collagen type IV, but can only migrate on collagen type IV (Tucker et al., 1990, Coplen et al., 1991).

In normal human bladder urothelium both the $\alpha 2$ and $\alpha 3$ subunits of the integrin protein are found associated with the ß1 subunit while in invasive bladder tumours only in

24% of the samples the $\alpha 2$ subunit has been observed. The $\alpha 1$, $\alpha 3$, $\alpha 4$ and $\alpha 5$ subunits are expressed in invasive tumours. A difference in migration of the tumour cells compared to the normal urothelium could occur due to a different pattern of integrin expression (Liebert et al., 1994).

General properties of growth factors

Growth factors are (soluble) polypeptides, with the ability to induce biological responses. Most mammalian cells secrete a variety of growth factors and respond to growth factors in an autocrine or paracrine way. Growth factors can bind to specific transmembrane receptors. Subsequently, cytoplasmic proteins are activated to mediate a variety of cellular responses (Chao, 1992, Hsuan, 1993, Sa et al., 1994). These responses include: cell proliferation, differentiation, apoptosis (programmed cell death) and motility (Cross et al., 1991, Pusztai et al., 1993).

Growth factors are classified into distinct families based on structural resemblance of the peptides. Epidermal growth factor (EGF) and fibroblast growth factor (FGF) belong to two different families. EGF belongs to the epidermal growth factor family. The proteins of this family all have, at the same positions, 6 cysteine residues. FGF belongs to the fibroblast growth factor family and several of these proteins show a high affinity for heparin or heparin sulphate chains. At this moment EGF and FGF seem to be the most relevant in urothelial physiology and carcinogenesis. EGF is a 6 kD protein which is secreted by most murine and human epithelial cells (Carpenter, 1979). Transforming growth factor α (TGF α) belongs to the same family. TGF α and EGF bind to the same receptor with almost the same affinity.

Acidic FGF (aFGF) and basic FGF (bFGF) do not have a signal sequence and their mechanism of secretion is unknown. However, these growth factors are known to interact with membrane bound receptors on target cells. Expression of bFGF cDNA in BALB/c 3T3 cells induces morphological transformation which could be inhibited by antibodies against bFGF. The bFGF induced transformation is therefore caused in an autocrine way (Sasca et al., 1988). Thus, aFGF and bFGF are secreted although not through the classical pathway.

Influence of growth factors on migration

In vivo studies in pigs have shown that growth factors in a cellulose gel placed onto a wound stimulate regeneration. A combination of growth factors (platelet derived growth

factor [PDGF]-2 and insulin like growth factor [IGF]-I or PDGF-2 and TGF α) enhance regeneration in the absence of a strong inflammatory reaction (Lynch et al., 1989). Schultz et al. (1992) demonstrated that EGF in tear fluid regulates the regeneration of cornea epithelium.

In vitro studies demonstrated that EGF and TGF α can influence the migration and proliferation of epithelial cells, keratinocytes and endothelial cells (Barrandon et al., 1987, Nickoloff et al., 1988, Schultz et al., 1991, Westermark et al., 1991). Addition of EGF to a wounded epithelial monolayer (mammary or kidney epithelium) stimulates migration and wound healing (Kartha et al., 1992, Matthay et al., 1993). EGF was also found to stimulate proliferation, migration and invasion of brain tumours cells into glioblastoma specimen (Engebraaten et al., 1993).

Acidic and basic FGF can influence proliferation, differentiation and migration of epithelial and endothelial cells. Gherardi et al. (1989) demonstrated that bFGF could increase movement of epithelial cells *in vitro*. Wound healing in *in vitro* monolayers of endothelial cells is stimulated by bFGF (Sato et al., 1988, Hoppenreijs et al., 1994). Mignatti et al. (1991) observed that bFGF is released by viable cells and thus might mediate migration via an autocrine mechanism. Thus, EGF, aFGF and bFGF are able to stimulate migration in various cell types.

Indirect influence of growth factors on migration and attachment

Migration and attachment of cells could also indirectly be influenced by growth factors. Growth factors may stimulate cells to produce extracellular matrix components. TGF ß perhaps represents the best known example of growth factors that can induce ECM production (reviewed in Haralson, 1993, Lawrence et al., 1994, Rogers et al., 1994). TGF ß stimulates collagen type III expression and diminishes the expression of collagen type I after radiation of the mammary gland (Barcellos-Hoff, 1993). Basic FGF stimulates the production of laminin in neuroepithelial cells (Drago et al., 1991). EGF can stimulate the production of collagen type IV in lung epithelial cells and fibronectin production by keratinocytes (Nickoloff et al., 1988, Federspiel et al., 1991).

Growth factors can also regulate the expression of integrin receptors and in this way they can also influence the migration of cells on ECM. For example, EGF stimulates the migration of cornea epithelium on fibronectin by increasing the number of fibronectin

receptors (Nishida et al., 1992a, 1992b). Basic FGF upregulates the $\alpha 2\beta 1$ receptor (laminin and collagen receptor) in endothelial cells which enhances the migration of these cells (Enenstein et al., 1992).

Growth factors in the urinary bladder

In man, mouse and rat a high concentration EGF is found in urine (Starkey et al., 1975, Hirata et al., 1979, Yura et al., 1989). EGF is only expressed in the superficial cells of the normal urothelium. Similarly, in bladder carcinoma cells EGF expression is found. The expression of EGF is inversely correlated with tumour grade (Lau et al., 1988). Both *in vitro* and *in vivo* it has been demonstrated that EGF enhances urothelial tumour growth and proliferation (Dubeau et al., 1987, Kuranami et al., 1991, Momose et al., 1991, De Boer et al., 1993, Kawamata et al., 1993). The EGF receptor is exclusively present in the basal cells of the normal urothelium (Messing, 1990). An increased expression of EGF receptors is found in the more aggressive TCC (Neal et al., 1985, 1989, Lipponen et al., 1994).

A possible explanation for the finding that EGF, although present in the urine, does not stimulate the proliferation of normal urothelium is the limitation of EGF receptor expression to the basal cell layers. The urothelium has a well-developed barrier structure on the surface that prevents passage of urine components, including EGF (Hicks, 1975). Injured urothelium, however, can allow EGF in the urine to reach the basal cell layer and stimulate these cells to proliferate.

In urine of mice and humans with bladder cancer increased levels of aFGF and bFGF are present (Chodak et al., 1986, 1988) compared to urine of normal individuals. In bladder carcinoma cells aFGF has been found and its expression appears to increase with tumour grade (Ravery et al., 1992). Acidic and basic FGF have mitogenic effects on NBT II rat bladder carcinoma cells. The capacity of these NBT II cells to invade through the muscle layer in bladder explant cultures is enhanced by aFGF (Vallés et al., 1990, Tucker et al., 1991). Allen et al. (1993) showed that bFGF is expressed in invasive human bladder carcinoma cell lines and not in non-invasive bladder cell lines. Thus, increased levels of aFGF and bFGF are found in bladder carcinoma cells and in urine of patients with bladder carcinomas. These growth factors may promote tumour invasiveness.

General properties of cell adhesion structures

Cell junctions are defined as specialized structures between two cells. Both cells equally contribute to these junctions. Cell-cell junctions perform the following functions: a) strong structural link between cells b) communication between cells by connecting the cytoplasmatic domains c) electrochemical impulse connections d) seal between the cells to form a selective barrier. The last function is mostly performed by tight junctions. Gap junctions mostly account for the communication between cells. Structural links between cells are mostly mediated by desmosomes and homophylic adhesion proteins.

The desmosome (macula adherens) is considered to be the prototype of a cell-cell adhesion structure. Desmosomes connect intermediate filaments from one cell to an other. Desmosomes are composed of desmogleins and desmoplakins. Desmogleins are a series of transmembrane glycoproteins localized in the intercellular space. The desmoplakins are proteins residing in the cytoplasmic plaque (Pauli et al., 1989, Buxton et al., 1993). Other groups of proteins that maintain the cell-cell adhesion are the "adhesion molecules". The cadherins are molecules that perform a homophylic adhesion, in which one protein specifically binds to an identical protein on a neighbouring cell. As long as the cadherin-cadherin interaction is functionally intact, inactivation of other cell-cell adhesion receptor molecules has little effect on cell-cell adhesion (Duband et al., 1987). Therefore, cadherins seem to represent the most important adhesion receptor molecules for the formation of physical cell-cell association. The other cell-cell adhesion molecules like the integrin superfamily, immunoglobulin-like superfamily and selectins are beyond the scope of this thesis.

Cadherins

Cadherins are cell surface glycoproteins which are calcium dependent for their cell-cell adhesion function. Cadherins are important for maintaining intercellular connections. They are divided into subclasses with a common basic molecular structure. By molecular cloning of cadherin cDNA it has been shown that cadherins are integral membrane proteins with a length of 723-748 amino acids and displaying 50% homology between the subclasses (Gallin et al., 1987, Hatta et al., 1988, Bussemakers et al., 1993). There are three major cadherin subclasses: E-cadherin (epithelial cadherin also known as uvomorulin and L-CAM); P-cadherin (placental cadherin) and N-cadherin (neural cadherin). Homologous cadherin

subclasses are generally well conserved across species (Heimark, 1990, Flemming, 1991, Takeichi, 1991, Pignatelli, 1993). Cadherin expression is found only at the intercellular borders within the same tissue.

The mechanism of cadherin function involves specific homophylic binding of extracellular domains at the cell surface on the one hand and their interaction with catenins in the cytoplasm on the other hand. In turn catenins are connected to actin filaments. The α, ß and γ catenins form a complex which interacts with the conserved cadherin cytoplasmic domain. The α catenin probably associates with the cadherin and with the ß and γ catenin and the α catenin binds also to actin filaments. The ß catenin is involved in the signal transduction, but how the ß catenin induces signal transduction is not known yet. The function of γ catenin is not known yet. The protein seems to be a plakoglobin, a component in the desmosomal plaque. Thus the cell-cell adhesion of cadherins is regulated by a family of catenins which regulate signal transduction and interactions with the cytoskeleton (Gumbiner et al., 1993, Piepenhagen et al., 1993). If the catenin function is impaired the E-cadherin mediated cell-cell adhesion is also lost (Shiozaki et al., 1994).

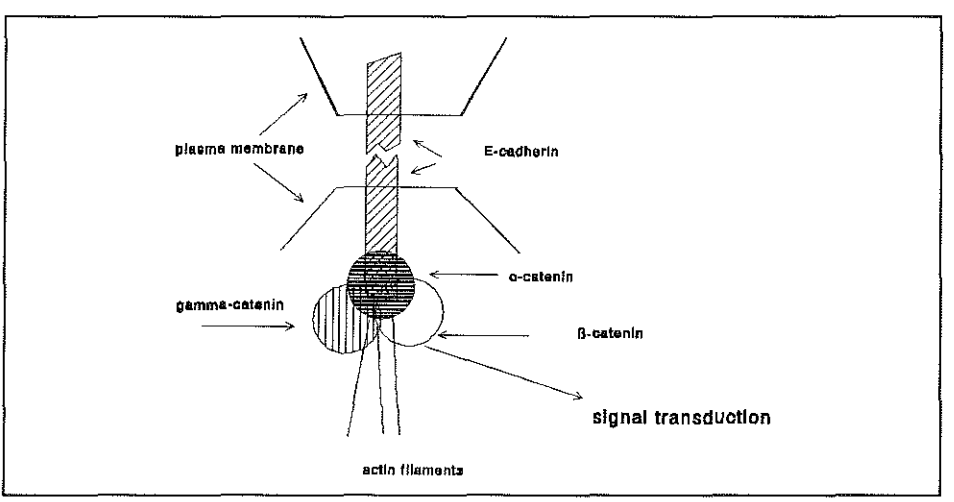

Figure 4-Proposed model for Cadherin-catenin protein complexes in cells (Figure adapted from Gumbiner et al., 1993)

Transfection studies with cDNA of E-cadherin or P-cadherin revealed a cell sorting effect. When L-cells (lacking cadherin expression) were transfected with either E or P-cadherin and then mixed, cellular aggregates were observed composed exclusively of cells with the same cadherin subclass expression (Nose et al., 1988). Cell sorting of animal cells

derived from different cell types was already described by Townes and Holtfreter in 1955. When different cell types are mixed in culture these cells sort them self into separate aggregates exclusively composed of their tissue origin. Cadherins can thus play an important role in this cell sorting process.

Cell lines lacking E-cadherin expression show invasive properties in *in vitro* assays, whereas E-cadherin positive cells do not show this feature (Frixen et al., 1991). When the cDNA of E-cadherin was transfected into invasive L-cells or epithelial tumour cell lines the cells lost their capacity to invade in *in vitro* invasion assays. Transfection of non-invasive E-cadherin positive cell lines with an E-cadherin specific anti-sense RNA endowed these cells with the capacity to invade. Similarly, an antibody against E-cadherin could induce invasiveness in a non-invasive E-cadherin positive cell line (Chen et al., 1991, Vleminckx et al., 1991). Thus, E-cadherin appears to be an adhesive molecule responsible for cell sorting, and acts as an invasion suppressor molecule.

In vivo, in highly invasive tumours such as adenocarcinomas of the prostate and squamous cell carcinomas of head and neck a loss (or down regulation) of E-cadherin expression is found. This loss of E-cadherin correlates with tumour grade (Umbas et al., 1992, Doki et al., 1993, Mathijssen et al., 1993, Mayer et al., 1993, Otto et al., 1993, Pizarro et al., 1994). In an immunohistochemical study Moll et al. (1993) showed in infiltrating lobular breast carcinomas, characterized by dispersed invading neoplastic cells, a total loss of E-cadherin expression. In ductal carcinoma of the breast, consisting of adhesive sheets and strands of tumour cells, E-cadherin expression was found. The above *in vivo* examples demonstrate that E-cadherin expression strongly influences the morphology and growth pattern of a carcinoma.

Cadherins in the urinary bladder

In the urothelium, E-cadherin expression is found only at the cell-cell borders as in other epithelial tissues. At the site of the basal urothelial cells that is in contact with the basement membrane no E-cadherin molecules are found (Bringuier et al., 1993). Two non-invasive human bladder carcinoma cell lines (RT 4, RT 112) express E-cadherin while the invasive bladder carcinoma cell lines J28 and T24 do not express E-cadherin (Frixen et al., 1991). Bringuier et al. (1993) demonstrated that *in vivo* in human bladder carcinoma a correlation exists between patient survival and E-cadherin expression. Out of 23 invasive

tumours 19 tumours lacked E-cadherin staining of the cell-cell borders. Decreased E-cadherin expression correlates with shorter survival in cases of invasive bladder tumours. In a recently published paper by Otto et al. (1994) essentially the same results were obtained on a larger series of TCC. Whether a relation exists between E-cadherin mediated cell-cell adhesion and the high recurrence rate in bladder cancer has not been studied yet.

Conclusion

In conclusion, migration of cells could be influenced by ECM components, growth factors or growth factor mediated expression of ECM components and or ECM receptors. The role of growth factors or ECM components on the intra-epithelial spread of transformed cells is unknown. Since these factors have an effect on migration of both tumour cells and normal regenerating epithelial cells it is very likely that they might be involved in intra-epithelial expansion.

Cadherins belong to the category of homophylic adhesion molecules and influence the morphology and invasive properties of a cancer cells. It is conceivable that cadherins play a role in the attachment of shed bladder tumour cells onto intact urothelium. This cellular attachment is an important perquisite for the process of tumour recurrence. In this thesis the involvement of growth factors, ECM components and cadherins in bladder tumour recurrence is explored.

1.5 SCOPE OF THIS THESIS

In the Netherlands the incidence of bladder cancer among men is 24 out of 100,000 and among females 7 out of 100,000 a year. The major problem in the management of patients with bladder cancer is the multifocality and the high recurrence rate after local resection of the tumour. The mechanisms underlying the high recurrence rate of bladder cancer are not clear as yet. Presumably, a proportion of the tumour recurrences develop from morphologically identifiable precursor lesions. We hypothesized that the intra-epithelial expansion of neoplastic cells at the expense of normal urothelium could be an important factor determining the recurrence incidence of bladder cancer. This hypothesis was corroborated by the observations that most tumour recurrences are of monoclonal origin and should therefore be considered the progeny of a single transformed cell. The research described in this thesis is an attempt at defining which factors could perhaps influence the bladder tumour recurrences. In order to investigate this we have developed an in vitro cocultivation system which allows outgrowth of murine bladder explants on transparent cyclopore membranes. The bladder outgrowth closely mimics the *in vivo* situation as multilayering and differentiation into umbrella cells occur. In chapter 2 a cocultivation in vitro model is described that allowed us to study the intra-epithelial expansion of tumour cells into the urothelium. The role of growth factors and extracellular matrix components on the expansion of urothelium is described in chapter 3. In chapter 4 and 5 the effects of extracellular matrix components, growth factors and cell-cell adhesion on the intra-epithelial expansion of tumour cells into the primary urothelium is described. In chapter 5 and 6 an other pathway of tumour recurrence is described being the attachment of shed tumour cells to intact or traumatized urothelium. In chapter 5 we focused our attention on the influence of cell-cell adhesion molecules on the attachment of tumour cells to intact urothelium. In chapter 6 the attachment to and colonization of tumour cells on traumatized urothelium and the effects of growth factors on this process will be discussed.

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CHAPTER 2

AN IN VITRO MODEL OF INTRA-EPITHELIAL EXPANSION OF TRANSFORMED UROTHELIAL CELLS.

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International Journal of Cancer 54: 846-850; 1993

SUMMARY

Replacement of normal urothelium by precancerous epithelium may explain the high recurrence rate of human bladder cancer. An *in vitro* model was designed in order to study the mechanisms of expansion of transformed urothelial cells at the expense of normal urothelium. For this purpose mouse bladder explants were allowed to expand on a transparent porous membrane. Subsequently, cell sheets of the non-tumorigenic mouse urothelial cell line NUC-5, the tumorigenic mouse urothelial cell line NUC-5 Py or the human bladder carcinoma cell line T24 were inoculated adjacent to the primary explant. Daily measurements of the outgrowth of the bladder explant were performed, and all cultures were terminated on day 24. At this time the post-confluent primary urothelial cell outgrowths still showed proliferative activity, as demonstrated by bromodeoxyuridine incorporation. In due course the non-tumorigenic NUC-5 cells were replaced by the bladder outgrowth. T24 and NUC-5 Py cells were able to inhibit the bladder outgrowth, or even infiltrate or replace the explant. This was confirmed by immunohistochemistry with (species-specific) anti-cytokeratin antibodies and by microscopic evaluation of cross sections of the porous membrane. This co-cultivation model appears to be suitable for the *in vitro* study of the mechanisms of intra-epithelial expansion of transformed urothelial cell lines.

INTRODUCTION

Human bladder cancer is a common cancer in Western society, with high morbidity due to its high recurrence rate after local resection of superficial transitional cell carcinomas (Raghavan *et al.*, 1990). Exposure to carcinogens probably plays an etiological role in the pathogenesis of bladder cancer (Silverman *et al.*, 1989). Three possible mechanisms for the recurrence of human bladder cancer have been suggested: 1) *De novo* appearance of a malignancy of independent origin in the bladder (multifocal origin), based on the assumption that most urothelial cells would have been exposed to carcinogens, 2) monoclonal expansion of transformed urothelial cells replacing the normal urothelium by intra-epithelial neoplasia and 3) seeding of cancer cells on traumatized bladder wall during surgical removal of the carcinoma (Soloway and Masters, 1980). The latter two assumptions imply that the progeny of a single transformed cell leads to the tumour recurrences (monoclonal origin). Sidransky

et al. (1992) have demonstrated that the multiple simultaneously occurring bladder carcinomas in a single cystectomy specimen are of monoclonal origin. This observation supports the biological relevance of the latter two mechanisms.

(Infiltrating) bladder carcinoma is often surrounded by areas with precancerous urothelial lesions, i.e. atypia and carcinoma in situ (Koss *et al.*, 1974). This "field cancerization" may be the consequence of a replacement of normal urothelium by a clonal expansion of transformed urothelial cells. It is not yet clear by what mechanisms the (pre-)neoplastic urothelial cells replace normal urothelium. To obtain more insight into the process of intra-epithelial expansion of transformed urothelial cells an *in vitro* model is required that allows easy manipulation of growth conditions followed by morphological evaluation. For that purpose we intended to design a cocultivation model. Explant cultures of adult mouse bladder mucosa on porous membranes were used as an *in vitro* model for regenerating urothelium (Chlapowski and Haynes, 1979; Knowles *et al.*, 1983). On the porous membranes the outgrowths of primary bladder explants closely mimic the *in vivo* situation as multilayering and maturation to umbrella cells occurs. The intra-epithelial invasiveness into these explants by a non-tumorigenic mouse urothelial cell line (NUC-5), a tumorigenic mouse urothelial cell line (NUC-5 Py) and the human bladder carcinoma cell line (T24) were compared using morphometry and immunohistochemistry.

MATERIALS AND METHODS

Cell lines

Three urothelial cell lines were used in this study. NUC-5 is a spontaneously immortalized mouse urothelial cell line established from a bladder explant culture of a neonatal female C3H/Law mouse (Van der Kwast *et al.*, 1989). In this study the 25th to 28th passage of NUC-5 cells were used. NUC-5 Py is obtained by co-transfection of NUC-5 cells with p48.19, a plasmid which contains a linearized polyoma virus genome with a defective origin of replication (kindly provided by Dr G.M. Veldman) and a construct bearing the neomycin resistance gene (PK0) (Van Doren *et al.*, 1984).

NUC-5, NUC-5 Py and T24 were tested for tumorigenicity by subcutaneous inoculation of 6.10^6 cells in nude BALB/c mice and/or syngeneic C3H/Law mice. No tumours arose in NUC-5 inoculated nude mice within two months. Within 3 to 4 weeks tumours developed in nude and syngeneic mice when NUC-5 Py was used for inoculation. T24 is a human bladder carcinoma cell line obtained from M. Wigler. This cell line does not induce tumours in nude mice, which was also demonstrated by others (Flatow *et al.*, 1987).

Standard conditions of cell culture

NUC-5, NUC-5 Py and the primary explants on porous membranes (Falcon) were cultured in a 1:1 mixture of HAM's F10 medium and Dulbecco's modification of Eagle's medium (DMEM) obtained from Flow Laboratories (Zwaneburg, The Netherlands) supplemented with 10% heat inactivated foetal calf serum (FCS), 10 μg/ml insulin, 5nM/ml selenite, and 5 μg/ml transferrin as well as 50 nM 4-hydrocortisone-21 hemisuccinate, 2 mM L-glutamine, 100 I.U./ml penicillin and 100μg/ml streptomycin obtained from Sigma (St.Louis, MO, U.S.A.). This medium is termed standard medium.

T24 cells were propagated to confluence in DMEM supplemented with 5% FCS, 100 I.U./ml penicillin and 100 μg/ml streptomycin.

To obtain cell sheets the cells were grown to a confluent layer in a Falcon tissue culture flask and the bottoms were scraped with a disposable policeman. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2. Passage of confluent cell cultures was performed by incubation with 0.05% trypsin and 0.02% EGTA in Hank's balanced salts solution (HBSS) for 3 min at 37°C after which the cells were resuspended in standard medium. The medium of the porous membrane cultures was changed every other day with standard medium.

Cell transfection

Plasmids (p48.19, pK0) were applied to NUC-5 cells by the calcium phosphate precipitation method (Chen and Okayama, 1987). A total of 1.10^5 NUC-5 cells were transfected in a 60 mm dish with 5 μg p48.19 and 2.5 μg pK0. At 24 hr posttransfection the medium was changed and after another 24 hr 400 μg/ml G418 (GIBCO BRL, Breda, The Netherlands) was added to the medium for selection of drug resistant cells. Selection medium was changed twice weekly, and after 4 weeks colonies of G418 resistant cells were visible macroscopically. These colonies were separately cultured and screened by northern blot analysis for expression of Py virus early region containing the transforming genes (data not shown).

Bladder explant culture on porous membranes

Six to eight-week-old female C3H/Law mice were sacrificed and their urinary bladder was removed aseptically. From these bladders the mucosa was stripped from the underlying muscle layer, cut into two pieces, and each half placed on a transparent porous membrane (Falcon cell culture insert, Cyclopore membrane with a diameter of 25 mm; pore size 0.45 μm). The lamina propria of the explant was always in contact with the membrane. The lower compartment of the cell insert was then filled with 2.5 ml standard medium. After half an hour the bladder explant was covered with 2 ml standard medium. Epithelial outgrowths of the bladder were visible after one night in 80% of the cases.

For the cocultivation studies sheets of NUC-5, NUC-5 Py or T24 cells (total area of the cell sheet approximately 5 mm^2 or 1% of the surface of the porous membrane) were juxtaposed at each side of the bladder explants after removal of the medium in the upper compartment of the cell insert. The cell sheets were allowed to attach to the porous membrane for 2 hr and subsequently the upper compartment was refilled with standard medium. Cocultivation was always started on day 4 after initiation of the explant cultures. All cultures were terminated on day 24. Two hours before termination of the cultures bromodeoxyuridine (BrdU) was added (40 μg/ml). Cultures were fixed in 70% ethanol.

Morphometry

The surface of the bladder outgrowth was monitored daily through a light microscope.

From these overviews the surface area was drawn by hand daily and the expansion of the outgrowth was calculated. The surface area as determined in mm^2 was related to the total surface of the porous membrane (i.e. 465 mm^2). The explant outgrowth is always given as a percentage of the total surface of the porous membrane. When the outgrowth covered the whole membrane 100% outgrowth was reached. Only well growing explant cultures were used for the experiments. In practice this meant that at day 4 at least 8.5% of the membrane should have been covered. Data given in this study were derived from a pool of 3 different experiments to ensure adequate comparisons.

Reagents for immunohistochemistry

Monoclonal antibody RCK108 (specific for human cytokeratin 19) was obtained from Prof. Ramaekers (University of Maastricht, The Netherlands) and NCL 5D3 (specific for mouse and human keratin 8) was commercially obtained from Eurodiagnostics (Apeldoorn, The Netherlands). Both antibodies were diluted 1:10 in phosphate buffered saline (PBS) prior to use. Monoclonal antibody against BrdU was also obtained from Prof. Ramaekers. This antibody was diluted 1:100 in PBS. As secondary antibody, a rabbit anti-mouse immunoglobulin (Ig)-peroxidase conjugate obtained from DAKO (Etten-Leur, The Netherlands), was used. This antibody was diluted 1:100 in PBS. Diaminobenzidine (DAB) was used as chromogen. The DAB solution was prepared by dissolving 75 mg DAB tetrahydrochloride (Fluka, Oud-Beyerland, The Netherlands) in 100 ml PBS and 50 μl 30% hydrogen peroxide was added as substrate.

Immunohistochemistry

Separate parts of ethanol fixed porous membranes were stained with RCK108 or NCL 5D3, and anti-BrdU. For BrdU staining half the membranes were stained following the procedure as described by Schutte et al. (1987). For cytokeratin staining the other half of the membranes were incubated with the primary antibody NCL 5D3 or RCK108 overnight at 4°C. Subsequently, an indirect conjugated peroxidase method was applied. For visualisation of bound antibody the membranes were incubated for 7 min with DAB solution and the sections were washed in distilled water. All membranes were counterstained with Mayer's haematoxylin.

To evaluate the position of immunostained (transformed or primary) urothelial cells more accurately the immunostained porous membranes were dehydrated in alcohol and embedded in paraffin, and cross sections perpendicular to the surface were made.

RESULTS

Primary bladder explant outgrowth on porous membranes

Control explant cultures usually had grown to confluency at day 18. Daily measurements of the surface area covered by urothelium showed a steady, consistent increase with time (Fig. 1a). From the immunostaining results with anti-BrdU antibody it is clear that at day 24 the post-confluent outgrowth still contained replicating cells (Fig. 2a). An intense labelling for cytokeratin 8 (NCL 5D3) was apparent in the urothelial cells of the outgrowth.

From cross sections it becomes clear that in some areas three distinct cell layers are present, similar to the mouse bladder urothelium *in vivo* (Fig. 2b).

Syngeneic cocultivation model

Sheets of the non-tumorigenic mouse urothelial cell line NUC-5 or its tumorigenic counterpart NUC-5 Py were inoculated on the porous membrane on day 4 after onset of the primary bladder explant. The edges of the explant outgrowth were well demarcated throughout the experimental period. This permitted daily measurements of the surface covered by the (expanding) explant, despite the presence of NUC-5 or NUC-5 Py. By day 7 the bladder outgrowth and the inoculated cell lines were contiguous (Fig. 3). The 6 bladder explants cultured in the presence of NUC-5 expanded to confluency at approximately the same rate as the controls (i.e. within 20 days) (Fig. 1b). At day 24 remaining NUC-5 cells were hardly detectable. In contrast, cocultures with NUC-5 Py cells showed a more variable pattern. A significant inhibition of explant outgrowth was observed in all cases, but in only three cases the explant cultures were largely replaced by NUC-5 Py (Fig. 1c). In the remaining 5 cases the bladder explants were able to expand slowly after an initial plateau phase of about 10 days.

To distinguish between explant cells and cells from the NUC-5 cell lines immunohistochemical staining with NCL 5D3 (keratin 8) was performed on 24 day explant cultures cocultivated with either NUC-5 or NUC-5 Py. NCL 5D3 was applied since this antibody selectively reacts with primary urothelial cells, but not with NUC-5 or NUC-5 Py cells. Cross sections revealed that the outgrowth of bladder explants that were cocultivated with NUC-5 cells consisted only of NCL 5D3 stained epithelial cells. However, in cross sections of explants cocultivated with NUC-5 Py cells we frequently observed a contiguous row of NCL 5D3 negative (i.e. NUC-5 Py) cells covered by NCL 5D3 positive (explant derived) urothelial cells (Fig. 4a). Occasionally, the unstained cells below the bladder outgrowth formed multilayers which were covered by some flattened NCL 5D3- positive cells (Fig. 4b). Where no bladder outgrowth was present NUC-5 Py cells formed unstained multilayers with a striking nuclear polymorphism. Immunostaining for BrdU revealed that at day 24 both the (remaining) primary explant outgrowth and NUC-5 Py cells contained cycling cells. The proliferative compartment of NUC-5 Py greatly exceeded that of the bladder outgrowth (data not shown).

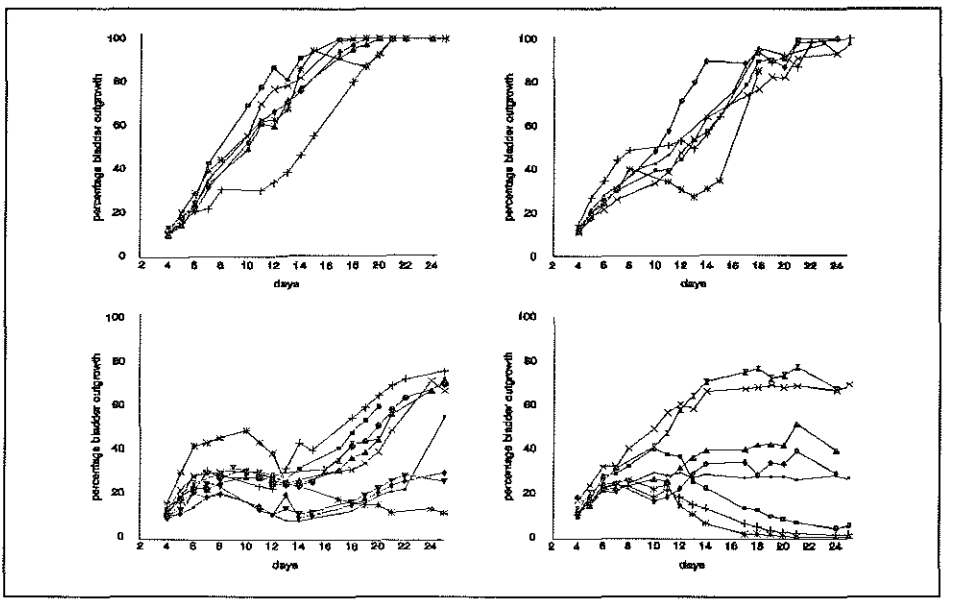
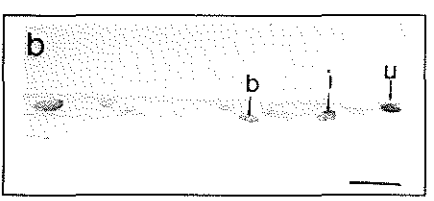


Figure 1-Influence of cocultivation of (transformed) bladder cell lines on the growth of bladder explants. Each line reproduces the outgrowth of one explant culture. (a) Control; (b-d) Growth of the explant when cocultivated with NUC-5, NUC-5 Py and T24 respectively.



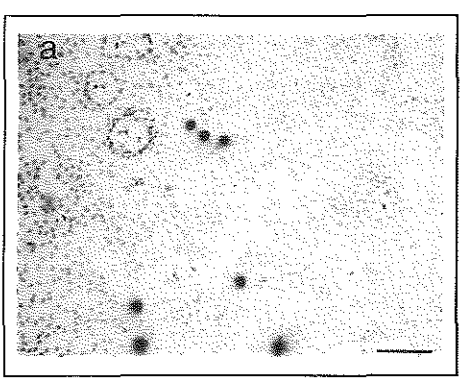


Figure 2- Micrographs of a control bladder explant after 24 days. (a) Overview of a BrdU labelled control explant culture on a porous membrane. Dark dots represent (proliferating) BrdU-positive cells. Scale bar=20 μm. (b) Cross section of a representative part of the membrane on day 24 labelled with NCL 5D3. The membrane is at the bottom, scale bar=10 μm. (u= umbrella cell with large cytoplasmic extension, i= intermediate cell and b= basal cell)

Xenogenic cocultivation model

During the first three days after inoculation of T24 cells the explants expanded until they touched upon the growing T24 cells. The T24 cells formed a monolayer which continued to grow during the course of the experiment. In the majority of the cocultivation

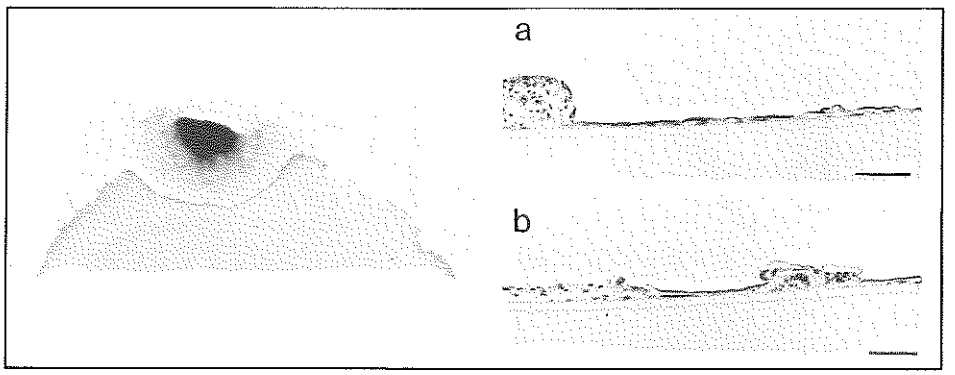


Figure 3- Low-power overview of a bladder explant culture on day 7. Cocultivated cells were NUC-5 Py. The culture was stained with NCL 5D3 and nuclei were counterstained with haematoxylin.

Figure 4- Cocultivation of bladder explant and NUC-5 Py cells. Cross sections of the culture were stained with antibody NCL 5D3. Scale bar=20 μm. Porous membrane is at the bottom. (a) NUC-5 Py cells (not stained) grown in a multilayer. A monolayer of NUC-5 Py cells is visible underneath the immunopositive primary bladder cells. (b) Cross section from another part of the culture. NUC-5 Py grown in multilayers underneath the primary immunostained bladder cells.

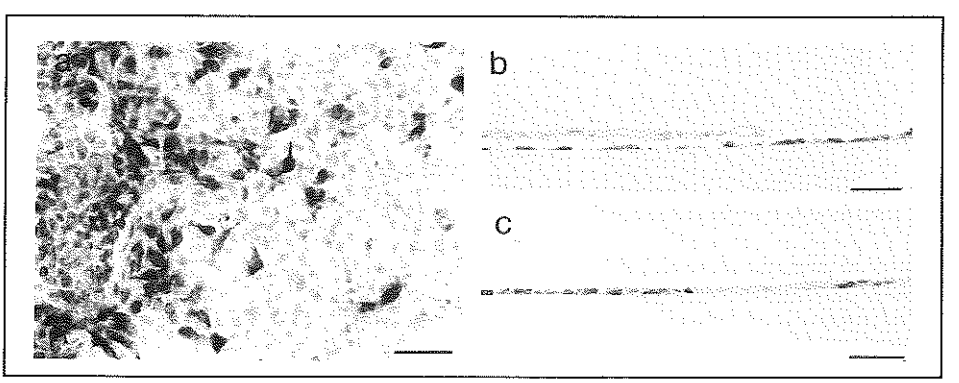


Figure 5- Cocultivation of bladder explant and T24 cells. Membranes were stained with antibody RCK108. (a) Overview demonstrating cytokeratin-positive T24 cells that infiltrate the primary bladder (not labelled). Scale bar=20 μm. (b) Cross section of the transition zone of T24 and primary bladder outgrowth. A monolayer of T24 cells (stained) is visible underneath the primary bladder culture (not stained). Membrane is at the bottom, scale bar=20 μm. (c) Cross section from another part of the culture. Immunostained T24 cells penetrating the primary bladder outgrowth. Scale bar=10 μm.

experiments T24 cells succeeded in occupying most of the surface of the porous membrane thereby inhibiting the growth of the explant as is shown in Fig. 1d. In 2 explant cultures T24 cells were only able to block the outgrowth of the bladder explant by day 12 at about 70% of the membrane surface. At day 24 immunohistochemistry was performed using monoclonal antibody RCK108. Previous experiments had shown that this antibody which is directed against human cytokeratin 19 did not react with murine epithelia. Therefore this antibody was used to identify T24 cells of human origin amidst the mouse bladder explants' urothelium. Both in overview and in cross sections it was apparent that individual RCK108 positive cells infiltrated the mouse urothelium (Fig. 5a,b,c). Most of these cells were attached to the surface of the porous membrane and they were generally covered by (unstained) mouse urothelial cells. Remarkably, the size of the invasive T24 cells strongly increased. Immunostaining for BrdU showed that at day 24 both the T24 and the explant outgrowth contained cycling cells (data not shown).

DISCUSSION

The most common form of intra-epithelial neoplasia is seen through the light microscope as carcinoma in situ. Although this form of intra-epithelial neoplasia can be regarded as a precursor lesion of overtly infiltrating carcinoma, it may also represent the intra-epithelial propagation of tumour cells originating from an adjacent carcinoma. Another form of intra-epithelial spread of neoplastic cells is the pagetoid type characterized by penetration of individual neoplastic cells into otherwise morphologically normal statified epithelium. Although this phenomenon is most commonly observed in breast cancer this variant growth pattern may also accompany other carcinomas among others bladder cancer (Koss, 1975; Mahadevia *et al.*, 1986).

The cell-biological mechanisms underlying intra-epithelial expansion of (pre)neoplastic cells at the expense of normal epithelium have not as yet been studied to a great extent. Intravesical administration of the carcinogen methylnitrosurea into rat bladders leads within 4 months to intra-epithelial neoplasia which ultimately progresses to invasive cancers (Steinberg *et al.*, 1990). Although this *in vivo* model of bladder carcinogenesis may be suitable for studying early stages of bladder neoplasia the dynamic relationship between normal and neoplastic cells cannot be investigated. *In vivo* transurethral inoculation of mouse

or human bladder carcinoma cells in mouse bladders is reported to result into histologically non-invasive or invasive tumours (Soloway and Masters, 1980; Ahlering *et al.*, 1987). Generally, invasive growth of the inoculated tumour cells prevails in these models obscuring the intra-epithelial expansion. Moreover, in *in vivo* models daily monitoring of the outgrowth and easy manipulation of intra-epithelial invasiveness is not possible. Therefore we established an *in vitro* model for intra-epithelial neoplasia using cocultivation of transformed urothelial cell lines with a primary urothelial explant. We observed that both massive replacement by crowding out of the regenerating urothelium by transformed cells occurs and that the so-called pagetoid type of intra-epithelial expansion may be visible. The latter growth pattern was easily visualized during cocultivation of the human bladder carcinoma cell line T24 with the explant. In this xenogenic combination T24 cells could be selectively immunostained with an anti-cytokeratin antibody permitting an unequivocal identification of single cells infiltrating into the multilayered primary urothelium.

The dynamics of the primary explant outgrowth during cocultivation with transformed cells was easily quantified by daily measurements owing to the transparency of the porous membranes employed. From these measurements it appeared that NUC-5 Py and T24 were able to inhibit and even push aside primary urothelial outgrowth in 25% of the cases. In 5 cases, however, the outgrowth continued to expand despite the presence of the transformed NUC-5 Py cell line. Cross-sections of cytokeratin stained membranes revealed that in the latter cases the morphometric analysis was deceptive as the transformed unstained cells (NUC-5 Py) were covered by a single cell layer of primary urothelium. From the above it is obvious that analysis of cross-sections is required to draw conclusions on cocultivation experiments. Additionally, the latter observation suggests that intra-epithelial expansion of sheets of transformed cells depends on their adhesion to extracellular matrix deposits of the primary bladder epithelium.

The finding that the spontaneously immortalized non-tumorigenic cell line NUC-5 is crowded out by the regenerating primary explant culture underlines the validity of our model for intra-epithelial neoplasia. Although NUC-5 cells are rapidly proliferating as judged from BrdU incorporation studies (data not shown) this cell line is not able to maintain itself opposite the much less rapidly proliferating primary urothelium. In addition in cytokeratin-stained cross sections of explants cocultivated for 24 days with NUC-5 cells, the presence of NUC-5 cells was not detectable anymore. Obviously NUC-5 cells lack some hitherto

unknown properties, that enable them to resist the expanding primary urothelium. This feature makes this cell line fit for gene transfection studies by which genes can be identified that endow NUC-5 with the property of intra-epithelial expansion.

Most *in vitro* cancer cocultivation models are designed to study mechanisms of invasion into the stroma by penetration of various types of collagens (Mareel *et al.*, 1979; Tucker *et al.*, 1991). An exception is the study by Kramer and Nicolson (1979). They inoculated a great variety of tumorigenic and non-tumorigenic cells and cell lines on completely confluent endothelial cell cultures and followed their interactions with time-lapse phase contrast microscopy and electron microscopy. In general, highly malignant or highly invasive cells *in vivo* were capable of attachment, invasion and migration under endothelial cells *in vitro* by disruption of inter-endothelial junctions. Similarly, Tucker et al. (1991) added spheroids of tumorigenic rat NBT-II urothelial cells on bladder organ cultures and observed that these spheroids progressively replaced the urothelium at the site of contact with the bladder explant. After exogenous or autocrine stimulation of NBT-II cells a deeper infiltration of the bladder wall by NBT-II cells was always observed. In their model system intra-epithelial invasion of NBT-II cells in the bladder explant was not further investigated.

The here described *in vitro* model may be useful in dissecting the cell biology of intra-epithelial expansion of epithelial cell lines with different biological behaviour. Methods to enhance resistance of primary urothelium to invasion by neoplastic cells may be developed using this approach.

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CHAPTER 3

AN IN VITRO MODEL OF UROTHELIAL REGENERATION: EFFECTS OF GROWTH FACTORS AND EXTRACELLULAR MATRIX PROTEINS

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Journal of Pathology 175: 283-291; 1994

SUMMARY

Although the cellular turnover of resting urothelium is very low, its regenerative capacity is known to be outstanding. In organotypic mouse urothelial cultures closely mimicking the differentiation and multilayering of normal urothelium we examined the cell biological mechanisms underlying urothelial regeneration and the specific role of growth factors and several extracellular matrix components (ECM). Exposure to epidermal growth factor (EGF), acidic fibroblast growth factor (aFGF) and culture on laminin resulted into an enhanced expansion of the urothelium. Microscopy and assessment of proliferative activity revealed that enhanced urothelial expansion due to EGF could be attributed to increased proliferative activity and increase in cell numbers, while aFGF stimulated expansion must be considered the consequence of increased cellularity and migration. Laminin enhanced urothelial expansion was shown to be the result of spreading of the entire urothelial organotypic culture. This was associated with a considerable decrease in number of cell layers. A synergistic effect of growth factors and laminin was not found. This organotypic urothelial cell culture model seems to be very useful in studying strategies to improve urothelial regeneration.

INTRODUCTION

Human bladder cancer is a common cancer in Western society with considerable morbidity due to its high recurrence rate after local resection of superficial transitional cell carcinomas.[1] Clinical and experimental data indicate that a substantial proportion of tumour recurrences is the consequence of seeding of tumour cells on traumatized and denuded bladder mucosa.[2] It can be envisaged that variations in regeneration rate of traumatized urothelium can influence the incidence of tumour recurrence. The regenerative potential of urothelium after denudation of the bladder mucosa has been studied in several species, including men and mouse and is known to be outstanding:[3,4] Within 48 hours after stripping the urothelium the whole mouse bladder is again covered with urothelium.

In vitro studies on keratinocytes, thyrocytes and endothelial cells have suggested that growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF) and transforming growth factor ß (TGFß) can influence the migration of these cell types.[5-9] For

the NBT II rat bladder carcinoma cell line it has been shown that exposure to acid FGF (aFGF) led to an increased migration.[10]

Basement membranes of epithelium, including urothelium are composed of several extracellular matrix (ECM) components such as laminin (lam), fibronectin (FN) and collagen type IV (COL IV).[11,12] ECM-cytoskeletal interactions are also reported to be crucial in the regulation of regeneration and wound healing. For example, FN appears to enhance the migration of corneal epithelial cells following wounding[13] and laminin stimulated the migration of olfactory neuro-epithelial cells.[14] Since data on growth factor and ECM modulation of urothelial regeneration are scarce we engaged to study these aspects in an in vitro model using organotypic cultures of murine urothelium. For that purpose explant cultures of adult mouse bladder mucosa on transparent porous membranes, uncoated or coated with ECM, were used as an in vitro model for regenerating urothelium. The culture on transparent membranes allowed daily measurements of the circumference of the explant outgrowths. Previous studies from our laboratory on this culture system had revealed that the outgrowths of the primary murine bladder explants closely mimic the in vivo situation as multilayering and maturation to umbrella cells occurs.[15,16] Our approach contrasts with most previous in vitro studies in which the migration or cell spreading of single cells or cell lines was studied.

MATERIALS AND METHODS

Bladder explant culture on porous membranes

Six to eight week old female C3H/HE mice were sacrificed and the urinary bladder was removed aseptically. The mucosa was stripped from the underlying muscle layer, cut into two pieces and each half was placed on a transparent porous membrane (Falcon cell-culture insert, Cyclopore membrane with a diameter of 25 mm; pore size 0.45μm). The lamina propria of the explant was always in contact with the membrane as described previously.[15] The medium of the porous membrane cultures was changed every other day with standard medium (a 1:1 mixture of Dulbecco's modified Eagle's medium [DMEM] and HAM's F10 medium with 10% heat inactivated foetal calf serum [FCS] and supplement as described previously[15]). In some experiments growth factors were added to the standard medium beginning at day 4 (as indicated in the text). Growth factors used were: aFGF 20 ng/ml obtained from Boehringer Mannheim (Almere, The Netherlands); EGF 20 ng/ml and TGFß 1 ng/ml obtained from Sigma (St. Louis, USA). This growth factor supplemented medium was changed every other day.

The circumference of the bladder outgrowth was monitored daily through a light microscope. From these overviews the expansion of the outgrowth was determined. The

surface area as determined in mm^2 was related to the total surface of the porous membrane. When the outgrowth covered the whole membrane 100% outgrowth was reached. Only explant cultures exceeding an outgrowth of 8.5% on day 4 were included in the study to obtain only well growing cultures.

Two hours before termination of the culture 40 μg/ml bromodeoxyuridine (BrdU) was added.

Culture substrates

FN and laminin (Sigma, St Louis, USA) were diluted in phosphate buffered saline (PBS) to a concentration of 25 μg/ml. COL IV (kindly donated by Dr. J.P.M. Cleutjens of the Dept. of Pathology, University of Limburg, The Netherlands) and collagen type I (COL I) (Vitrinogen 100; ICN laboratories, Amsterdam, The Netherlands) were diluted in Ham's F10 to a concentration of 25 μg/ml. Porous membranes were covered with 0.5 ml of a solution containing one of the ECM proteins, and incubated overnight at 37°C in a humidified atmosphere. Coated membranes were then incubated with 2.5 mg bovine serum albumin (BSA) for 30 min at 37°C to block non-specific binding sites on the membrane. Explants on BSA coated membranes were regarded as negative controls, since BSA is not an adhesion molecule. Membranes were washed with H_2O and dried overnight. The next day the bladder explants were placed on the membrane.

^{3}H thymidine incorporation

When the primary bladder culture covered an area of 60% to 65% the standard medium was replaced by serum free DMEM with supplements containing 20 μCi ^{3}H thymidine/ml (1.25 ml/membrane) in which the urothelium was cultured for another 2 hours. Subsequently, the culture was rinsed with PBS with an excess of non-labelled thymidine. The incorporated radio-activity was counted in Ultima Gold (Packard, Groningen, The Netherlands) using an α,ß-scintillation analyzer (as described previously[16]). Before counting incorporated ^{3}H thymidine the membranes were immunohistochemically stained and counterstained with haematoxylin. To preserve an adequate morphology trichloroacetic acid (TCA) treatment of the membranes was omitted. Control experiments revealed no differences in ^{3}H thymidine incorporation values between membranes incubated and washed with 15% TCA and corresponding non-treated membranes.

Reagents for immunohistochemistry

Monoclonal antibody RGE 53 (specific for cytokeratin 18) was commercially obtained from Eurodiagnostica (Apeldoorn, The Netherlands). RGE 53 was employed to visualize the more mature urothelial cells. With this staining cells with a typical morphology of umbrella cells can be distinguished more easily. The monoclonal antibody against BrdU was obtained from Dr. Ramaekers (University of Maastricht, The Netherlands). For BrdU staining the ethanol fixed membranes were pre-treated with 1N HCL following the procedure as described.[17] The membranes were incubated with the primary antibody overnight at 4°C. Subsequently, an indirect conjugated peroxidase method was applied. A goat anti-mouse immunoglobulin (Ig)-peroxidase conjugate obtained from DAKO (Glostrup, Denmark), was used. Diaminobenzidine (DAB) was used as chromogen. The DAB solution was prepared by dissolving 75 mg DAB tetrahydrochloride (Fluka, Oud-Beyerland, The Netherlands) in 100 ml PBS and 50 μl 30% hydrogen peroxide was added as substrate. For visualisation of bound antibody the membranes were incubated for 7 min with DAB solution and the sections were washed in distilled water. All membranes were counterstained with Mayer's haematoxylin.

To evaluate the number of immunostained urothelial cell layers more accurately strokes of the immunostained porous membranes covered with urothelial cells were dipped in 5% gelatin, dried overnight in the air and embedded in paraffin. Cross sections (5μ) perpendicular to the surface were made. The remaining parts of the membranes were dehydrated in alcohol and embedded in Euparal (Chroma-Gesellschaft Stuttgart, Germany).

Quantification of cell density and cell spreading

To quantify the cell density of the urothelium the number of nuclei was counted in 14 random areas of 0.15mm^2 in the outgrowth explant cultures for the ^{3}H thymidine incorporation. At this time the bladder outgrowth covered about 60-65% of the membrane. The average was taken $\pm$ SEM out of 3 independent experiments with 2 samples each. In cultures covering approximately 100% of the supportive membrane the degree of cell spreading was evaluated by counting the number of basal nuclei on cross sections over a distance of 5mm. In each membrane 8 random positions at the peripheral part of the cultures were chosen for evaluation.

RESULTS

Modulation of expansion of the primary bladder outgrowth

In explant cultures kept in standard medium containing 10% FCS (control outgrowth) the urothelium immediately began to expand as a sheet of epithelial cells after attachment of the explants to the cyclopore membrane. The edges of the outgrowth of primary urothelium were well demarcated throughout the experimental period. This permitted an easy measurement of the surface covered by the expanding urothelium. On day 4 the urothelial outgrowth covered more than 8.5% of the membrane in 80% of the cases. The whole membrane was covered in approximately 22 days (100% urothelial outgrowth). Addition of EGF and aFGF into the standard medium containing 10% FCS led to an increase in expansion rate from day 7 onwards as compared to the controls ($p \leq 0.01$) (Fig. 1). Addition of TGFß resulted in a significant inhibition of the expansion from day 7 onwards.

The expansion rate of primary urothelium was also examined on membranes coated with different ECM proteins. No growth factors were added to the standard medium. In 95% of the cultures grown on ECM coated membranes the outgrowth was over 8.5% on day 4. Attachment percentage (40%) and expansion rate of explants on BSA coated membranes was significantly less than on uncoated membranes (Fig. 2). No significant difference in expansion rate was observed between urothelial cultures grown on COL IV, FN, or COL I coated supportive membranes and uncoated membranes (Fig. 2).

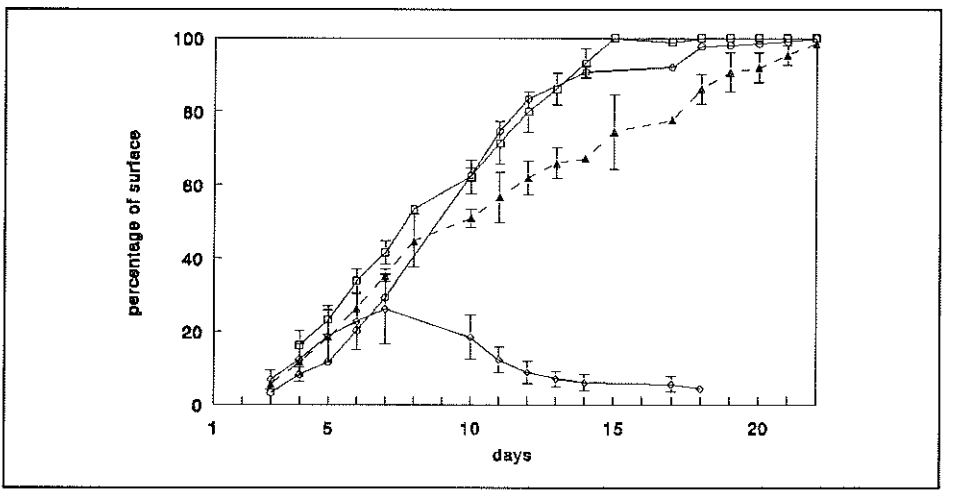

Figure 1- a) Influence of growth factors on the expansion rate of explant cultures. ○ EGF; □ aFGF; ◇ TGFß and ▲ control. Data are expressed as mean ± SEM of 3 independent experiments with 3 samples each.

Probably, constituents of FCS provide an adhesive substrate allowing the expansion of urothelial cells on uncoated membranes. However, the proportion of successfully attached explants was higher on the ECM coated than uncoated membranes (i.e. 95% versus 80%). The expansion of the urothelium cultured on laminin was increased from day 7 onwards as compared to the other cultures ($p \leq 0.01$).

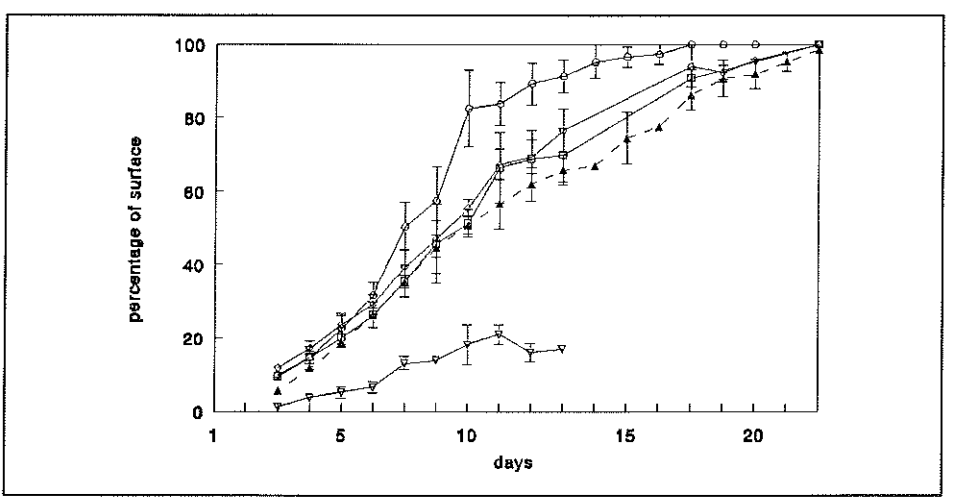

Figure 2- Influence of ECM on the expansion rate of explant cultures. ◇ FN; ○ laminin; ▲ control; ▽ BSA; □ COL IV. Mean ±SEM was calculated as explained in the legend of Fig 1.

Figure 3 gives the outgrowth of urothelial cultures grown on FN, laminin, COL IV, or COL I coated membranes in the presence or absence of EGF. Addition of EGF led to an increased expansion rate of urothelium on FN, COL IV, and COL I, but did not influence the expansion rate of urothelium grown on laminin.

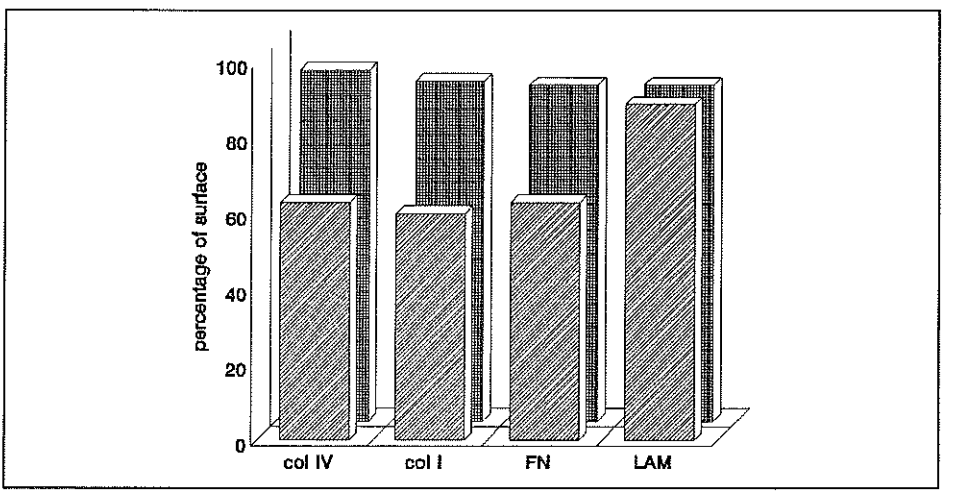

Figure 3- Influence of EGF on the expansion rate of explant cultures grown on ECM. Cultured for 11 days. Hatched bars represent explants cultured without EGF and dotted bars represent explants cultured in the presence of EGF

Modulation of multilayering and cellularity in the primary bladder outgrowth

After termination of the cultures, immunohistochemistry with antibody RGE 53 was performed in order to visualize maturation to umbrella cells. Cells with the typical morphology of umbrella cells were identified in control explant cultures, in cultures exposed to aFGF and TGFß and in urothelial outgrowths on all coated membranes without addition of growth factors. No RGE 53 positive cells with typical morphology of umbrella cells were found in EGF treated cultures. Furthermore, the exposure of urothelium on ECM coated membranes to EGF was associated with the disappearance of RGE 53 positive cells with the morphology of umbrella cells (Fig. 4a-c).

Cross sections of immunostained membranes (Fig. 5a-d) revealed that at near confluency EGF treated cultures had an increased thickness of 3-5 cell layers as compared to 2-3 cell layers in non-treated cultures. The cellularity increased from 179 $\pm$ 35 cells/0.15mm^2 in non-treated cultures to 414 $\pm$ 14 cells/ 0.15mm^2 in EGF-treated cultures. In aFGF treated cultures the cellularity was also increased to 280 $\pm$ 52 cells and the cultures

consisted of 2-3 cell layers with an upper layer of flattened RGE 53 positive umbrella cells. The TGFß treated culture consisted of one single cell layer of very large RGE 53 positive cells. Its cellularity decreased to 67 $\pm$ 1 cell/ 0.15mm^2.

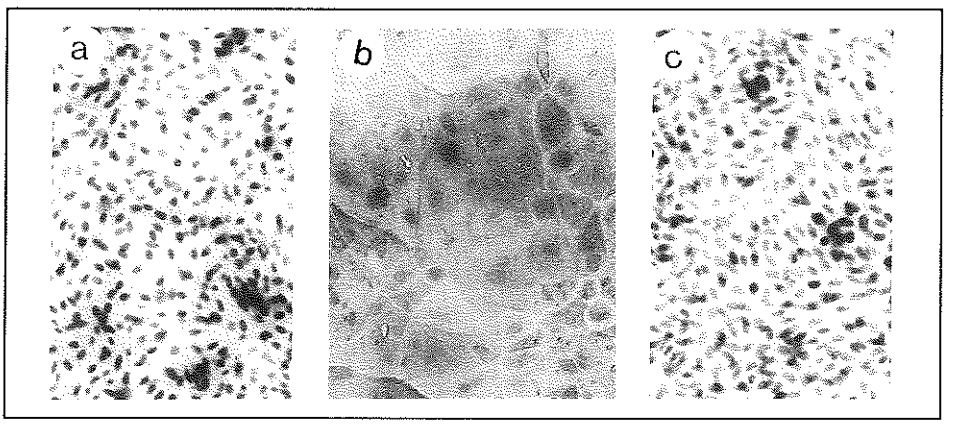

Figure 4- Overviews of the bladder explant after 20 days of culture. All cultures were immunohistologically stained with RGE 53, an antibody against cytokeratin 18 and counterstained with haematoxylin. (a) overview of an EGF treated culture. Note the absence of RGE 53 positive cells. (b) overview of a bladder explant cultured on COL IV. The cells have the typical morphological appearance of umbrella cells. (c) A bladder explant cultured on COL IV with addition of EGF. Note the absence of RGE 53 positive cells.

In cross sections of the urothelial outgrowths on COL I, COL IV or FN coated membranes (Fig. 5e-h) 2-3 cell layers were observed. The top layer of these cultures consisted of flattened RGE 53 positive cells with the morphology of umbrella cells. Urothelium on laminin coated membranes consisted of one cell layer of large flattened RGE 53 positive cells with a remarkably low nuclear density in the basal cell layer (table 1).

Cross sections of immunostained coated membranes showed that in the presence of EGF the number of cell layers and nuclear density of the basal cell layer (Table 1) was increased in all cultures irrespective of the coating (Fig. 5i-j).

Modulation of the proliferation of the primary bladder outgrowth

Table 1 shows that an increase in proliferative activity, as measured by ^{3}H thymidine incorporation at the time the culture reached a 60-65% outgrowth, was only observed in cultures exposed to EGF. Direct visualization of proliferating cells by immunostaining with anti-BrdU antibody revealed that these cells were evenly dispersed over the membrane. In contrast, 50% of the aFGF exposed cultures lacked these BrdU positive cells in the periphery

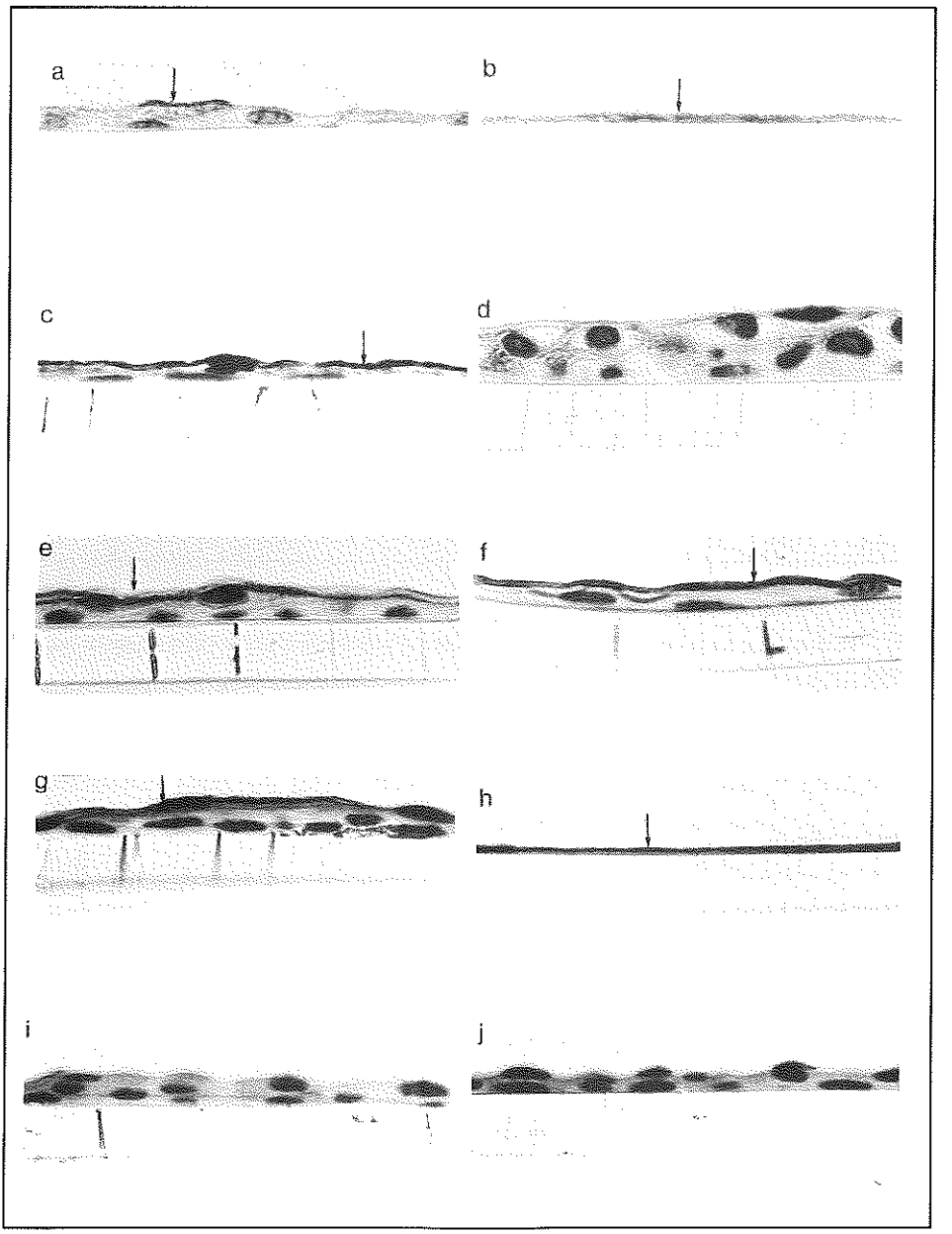

Figure 5- Cross sections of different urothelial cultures (x 1000). Supportive membranes are immediately below the urothelium. Arrows indicate the RGE 53 positive cells.
(a) control (b) TGFß culture (c) aFGF culture (d) EGF culture (e) grown on COL I (f) grown on FN (g) grown on COL IV (h) grown on laminin (i) grown on COL IV with addition of EGF (j) grown on laminin with addition of EGF

of the culture (data not shown).

	^{3}H thymidine incorporation DPM ± SD	number of nuclei in the basal cell layer ± SEM	number of cell layers
control	75,893 ± 10,550	7.7 ± 0.2	2-3
EGF	311,047 ± 3,790	15.3 ± 0.6	3-5
aFGF	72,154 ± 9,495	9.3 ± 0.8	2-3
COL IV	35,344 ± 18,602	9.3 ± 0.7	2-3
COL I	39,448 ± 15,102	7.9 ± 0.8	2-3
FN	32,577 ± 14,187	7.3 ± 0.6	2-3
LAM	20,007 ± 11,776	3.7 ± 0.8	1
COL IV + EGF	179,624 ± 36,791	13.1 ± 2.8	3-4
COL I + EGF	139,756 ± 3,788	13.4 ± 2.6	3-4
FN + EGF	103,313 ± 21,266	13.8 ± 3.1	3-4
LAM + EGF	130,460 ± 24,255	15.1 ± 2.4	3-4

Table 1- Proliferation, spreading of basal cells and number of cell layers in organotypic urothelial cell cultures.

Cultures on ECM coated membranes generally had a lower proliferative activity than the control. No difference in ^{3}H thymidine incorporation was found between the cultures when grown on different ECM coated membranes. Direct visualization of BrdU containing urothelial cells demonstrated proliferating cells in a peripheral concentric zone in 75% of the cultures.

Cultures grown on ECM components in the presence of EGF demonstrated an increase in ^{3}H thymidine incorporation compared to the primary urothelium grown on the ECM coated membranes in the absence of EGF (Table 1).

DISCUSSION

The high regenerative capacity of the bladder urothelium after acute damage is remarkable in view of the slow turnover of the normal transitional epithelium. Several mechanisms may account for the rapid re-epithelialization of the bladder surface 1) extension of the surfaces of remaining cells, 2) migration of cells out of the urethra and ureteral orifices and 3) an increase in proliferation of the (remaining) urothelial cells.[18]

In this study we presented evidence that each of these mechanisms was operating in our organotypic regeneration model either separately or in combination. In this organotypic culture system we studied the effects of growth factors or ECM components on the expansion of a cohesive sheet of primary urothelial cells instead of the effect of these factors on individual cells as most studies do. We demonstrated an increase in expansion rate of mouse bladder explants cultured either in the presence of EGF or aFGF, and when grown on laminin. Strikingly, the observed increase in expansion of the urothelium was accompanied by different morphological and functional features dependent on the applied culture conditions.

EGF stimulated urothelial expansion rate was associated with an increased number of cell layers, a higher cellular density as well as a loss of maturation into umbrella cells. The effect of EGF on expansion may thus be attributed to the observed strong increase in proliferative capacity. Previous studies on mouse and human epithelium also demonstrated an EGF-mediated stimulation of proliferation.[19-21] Similarly, Schultz et al.[22] showed that regeneration of corneal epithelial cells in vivo is regulated by EGF.

In urothelial cultures grown on laminin coated membranes the proliferative activity was decreased and at the peripheral parts of the culture the urothelium consisted of only one flattened cell layer with low nuclear density. The latter feature indicates an enhanced cell spreading of this cohesive sheet of cells. Laminin induced spreading of dispersed individual cells has been described for other cell types[23-25], but as yet not for organotypic cultures. It is most likely that the laminin-induced increase in urothelial expansion is due to this spreading of the urothelial cells.

To explain the mechanism by which aFGF induced an increased expansion rate is more complex. Addition of aFGF increased the cellularity of the urothelial culture, but at 60% of confluency the proliferation rate of the aFGF stimulated cultures was similar to the control cultures. Furthermore, in the peripheral edge of the aFGF stimulated cultures proliferating (BrdU positive) cells were largely absent. This suggests that proliferation does not greatly contribute to the aFGF induced urothelial expansion. More probably, migration rather than stretching or proliferation of the urothelial cells contributes to the observed aFGF enhanced expansion. This hypothesis is in line with the work of Valles et al[26] in which the authors showed that aFGF can serve as a migration factor in a rat bladder carcinoma cell line. Similarly, migration of urothelial cells may be involved in

the expansion of explants cultured on COL I, COL IV or FN. In these cultures proliferative activity is decreased, while the number of cell layers and the expansion rate remains equal to that of the cultures on uncoated membranes. Migration of epithelial cells on COL IV, COL I and FN was also found in other studies[27,28] but as yet not in organotypic urothelial cultures. The enhanced migration on ECM coated membranes of the urothelial cells is probably due to an interaction of ECM with ECM specific integrins.[29] Unfortunately, we were not able to detect $\alpha 3$, $\alpha 5$, $\alpha 6$, ß1 or ß4 subunits using immunohistochemistry.

The negative effect of TGFß on the expansion rate of the urothelium is probably due to the induction of maturation and apoptosis in urothelial cells[30] (De Boer et al. submitted).

Another question addressed by this study was whether growth factors can act synergistically with ECM in order to enhance expansion. Therefore, we cultured the urothelium on COL I, COL IV, FN or laminin and added EGF to the culture. Exposure to EGF led to a higher urothelial expansion on COl I, COL IV and FN coated membranes, but not on laminin coated membranes. Apparently, the mechanism of (laminin-mediated) cell spreading is not synergistic with the expanding effect of EGF mediated proliferation. Indeed, the high nuclear density of basal cells in EGF treated cultures on laminin coated membranes demonstrated that the laminin-mediated cell spreading is inhibited by exposure to EGF. EGF may have changed the integrin expression pattern in such a way that the laminin mediated cellular spreading is diminished. On the other hand, ECM coating of membranes reduced the EGF associated increase in number of cell layers but not the increased level of ^{3}H thymidine incorporation.

In conclusion, this in vitro mouse urothelium regeneration model has permitted the identification of separate factors that each selectively mediate the different mechanisms underlying regeneration of urothelium in vivo. We failed to show a synergism between the different mechanisms of regeneration. Future studies should indicate whether these factors may also operate in vivo during regeneration of normal urothelium after therapeutical denudation of urothelium.

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ACKNOWLEDGEMENTS

We wish to thank Mr F.L. van der Panne from our department for the photography. This study was supported by the Dutch Cancer Foundation (IKR 90-17).

CHAPTER 4

MODULATION OF INTRA-EPITHELIAL EXPANSION OF HUMAN T24 BLADDER CARCINOMA CELLS IN PRIMARY UROTHELIUM BY GROWTH FACTORS AND EXTRACELLULAR MATRIX COMPONENTS.

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International Journal of Cancer in press, 1994

SUMMARY

The high recurrence rate of bladder cancer is probably due to an efficient repopulation of the bladder by residual transformed cells after resection of the tumour. However, the regenerating capacity of the normal urothelial cells is very high. To study the balance between regenerating normal urothelium and outgrowth of transformed urothelial cells, we recently developed an in vitro cocultivation model. With this model system we studied the effects of growth factors and extracellular matrix components on the intra-epithelial expansion of human T24 bladder carcinoma cells in primary mouse bladder explants. Exposure of the cultures to acidic fibroblast growth factor (aFGF) and laminin led to a dramatic increase in number of invasive T24 cells into the primary urothelium. Epidermal growth factor (EGF), collagen type I and IV counteracted the infiltration of individual T24 cells. EGF, aFGF, laminin, collagen type I and IV did not directly affected the migration and proliferation of T24 cells. Apparently, the efficacy of invasion of transformed urothelial cells into primary urothelium is not only dependent on the intrinsic characteristics of the transformed cells, but can be influenced to a considerable extent by exogenous components which exert their influence on the normal urothelium.

INTRODUCTION

Over 70% of human bladder carcinomas are superficial carcinomas and only 15% of them progress to invasive tumours after infiltration through the basement membrane (Levi *et al.*, 1993). It is known that the frequency of tumour recurrence after local resection of the transitional cell carcinoma (TCC) of the bladder is very high and that these recurrent tumours may ultimately progress to invasive tumours (Heney *et al.*, 1983; Raghavan *et al.*, 1990). Originally it was thought that recurrent bladder carcinomas represented de novo tumours. However, it has recently been demonstrated that multiple simultaneously occurring bladder carcinomas, in a single cystectomy specimen as well as multiple tumour recurrences are of monoclonal origin (Sidransky *et al.*, 1992; Habuchi *et al.*, 1993). Thus tumour recurrences in the bladder most probably arise from residual neoplastic urothelial cells that replace the normal urothelium after therapy.

In a previous in vitro study we showed that transformed urothelial cells can indeed

replace primary urothelium (Rebel *et al.*, 1993). It is not yet clear by what mechanisms (pre-)neoplastic urothelial cells can replace the normal urothelium. Several findings suggest that growth factors could be involved in the growth of TCC. Epidermal growth factor (EGF) is found in high concentrations in normal urine (Hirata and Orth, 1979; Lau *et al.*, 1988). On the other hand, an increased amount of acidic fibfoblast growth factor (aFGF) and transforming growth factor α (TGFα) was found in urine of patients with bladder cancer compared to normal individuals. In addition, the expression of aFGF and basic fibroblast growth factor (bFGF) in TCC cells was higher than in normal urothelium (Kimball *et al.*, 1984; Chodak *et al.*, 1986; Chodak *et al.*, 1988; Ravery *et al.*,1992; Allen and Maher, 1993).

Intra-epithelial spread of transformed epithelial cells can be considered the consequence of cellular migration. Extracellular matrix (ECM) components can promote cell spreading and migration of different kinds of cells, e.g. during regeneration and wound healing (Nishida *et al.*, 1990; Calof and Lander, 1991). These combined data prompted us to consider growth factors and ECM components as potentially important factors which determine the level of intra-epithelial expansion of transformed epithelial cells.

In a previous study we showed that organotypic cultures on transparent cyclopore membranes are ideally suited to study the expansion rate and degree of differentiation of the primary bladder culture under influence of growth factors and ECM components (Rebel *et al.*, 1994). In addition, we described that the human T24 human bladder carcinoma cell line expanded into the primary urothelial outgrowth (Rebel *et al.*, 1993). In this paper we studied the influence of growth factors and ECM components on this process. The results obtained show that exposure to aFGF and coating of the supportive membranes with laminin stimulate the invasion of T24 cells. On the other hand, EGF treatment or coating with other ECM components counteracted the intra-epithelial T24 cell invasion.

MATERIAL AND METHODS

Bladder explant cultures on transparent membranes

Urinary bladders were obtained from six to eight week old female C3H/HE mice. The mucosa was stripped from the underlying muscle layer. Half of the bladder mucosa was placed on a transparent membrane (Falcon cell-culture insert, Cyclopore membrane with a diameter of 25 mm; pore size 0.45μm) with the lamina propria in contact with the membrane. The medium of the lower and upper compartment was changed every other day

with standard medium, that is a 1:1 mixture of Dulbecco's modified Eagle's medium and HAM's F10 with 10% heat inactivated foetal calf serum (FCS) and supplements as described previously (Rebel *et al.*, 1993: De Boer *et al.*, 1994).

When required growth factors were added to the standard medium: aFGF 20 ng/ml obtained from Boehringer Mannheim (Almere, NL) or EGF obtained from Sigma (St. Louis, USA). In other experiments the transparent membranes were pre-coated with 25 μg/ml fibronectin (FN), laminin (Lam), collagen type I (Col I) or with collagen type IV (Col IV) before the bladder explants were placed on the membrane as described (Rebel *et al.*, 1994).

For the cocultivation studies, two sheets of T24 cells of 5 mm^2 were inoculated on opposite sides of to the outgrowth (on day 4) of the bladder explant on day 4. The cell sheets were allowed to attach to the transparent membrane for two hours, subsequently the upper compartment was refilled with standard medium as described previously (Rebel *et al.*, 1993).

Immunohistochemistry

The membranes were fixed with 70% ethanol for 24 hours. Preincubation was done with 10% normal goat serum for 15 min, and then the membranes were incubated with the primary antibody RCK 108 overnight at 4°C. Subsequently, an indirect conjugated peroxidase method was applied as described before (Rebel *et al.*, 1993). All membranes were counterstained with haematoxylin, dehydrated and mounted. Monoclonal antibody RCK 108 (kindly provided by Dr Ramaekers, University of Maastricht, NL) is specific for human cytokeratin 19 and lacked cross reactivity with murine epithelia.

To evaluate the position of the immunostained T24 cells more accurately, strips of the immunostained transparent membranes covered with urothelial cells were dipped in 5% gelatin, dried overnight in the air and embedded in paraffin. Cross sections (5μm) were made perpendicular to the surface.

Quantification of the distance of lateral invasion

Quantification of the distance of lateral invasion of T24 cells in the primary bladder culture was done using an image analyzer (IBAS 2000 Zeiss Kontron, Oberkochen, FRG). The lateral invasion of T24 cells was visualized with a Hitachi CCTV camera and was analyzed with the supplied Kontron IBAS1 version 4.4 software program. The lateral infiltration distance of the T24 cells was the average of the most distant T24 cell found in the primary bladder culture on 30 random positions on the membrane.

Quantification of the number of invasive T24 cells was done by counting the number of T24 cells in 8 random fields of 0.15 mm^2 at the edge of the urothelial outgrowth of the bladder explant. For statistical analysis a student T-test was used.

Proliferation and expansion assays

To assess the effect of ECM components on proliferation of T24 cells, 1.10^3 T24 cells were seeded in 96 well-dishes which were coated with 25 μg/ml Lam, FN, Col I or Col IV. The effect of growth factors on the proliferation of T24 cells was investigated using non-coated dishes. On day 1 20ng/ml aFGF or EGF were added. After 5 days of culture 0.5 μCi/ml ^{3}H thymidine was added to each well and the cells were incubated for another 16 h. The cells were trypsinized and harvested. The ^{3}H thymidine incorporation was counted using an BetaPlate scintillator counter (LKB-Pharmacia, Woerden, NL) and expressed as cpm.

To asses the effects of ECM components and growth factors on the expansion of the T24 cells a sheet of T24 cells of 5 mm^2 was placed on a transparent membrane under the different conditions. The diameter of each T24 colony was measured every day. The

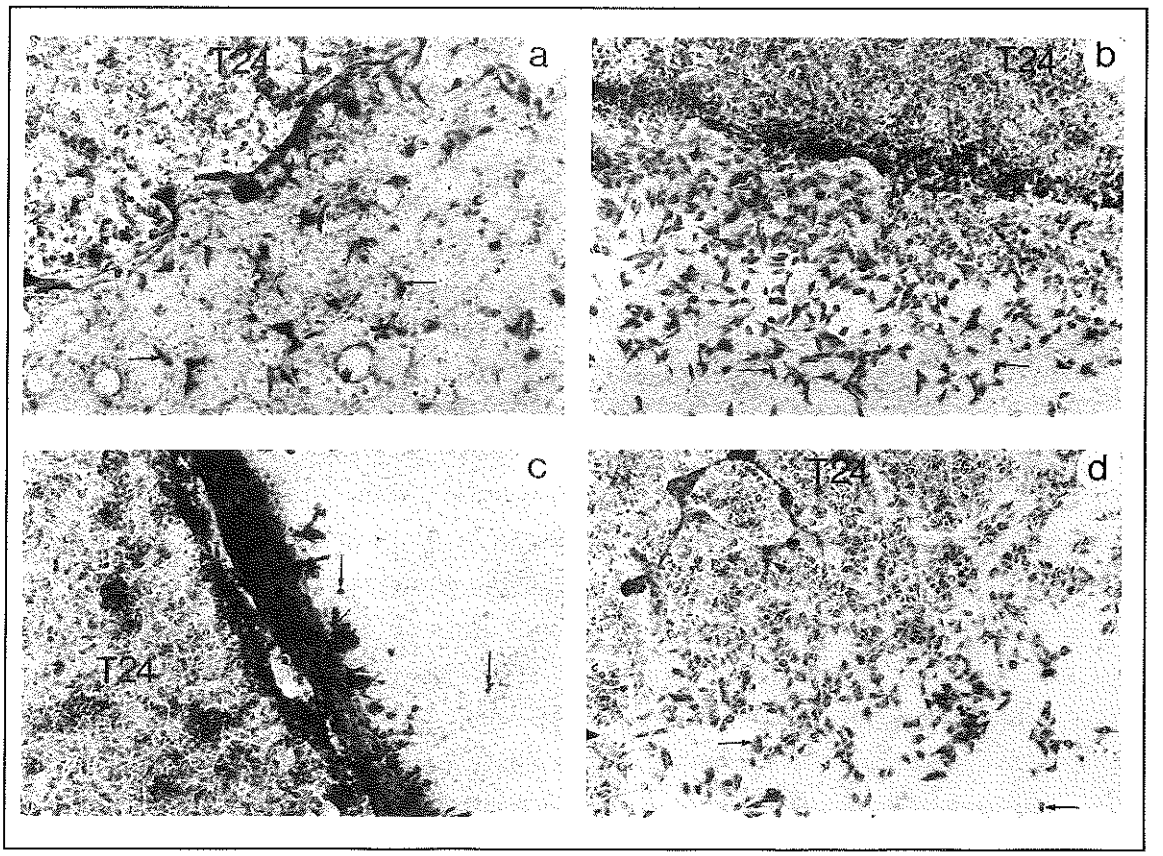
measurements made during the first 10 days were than used to calculate the speed of cell expansion. After 14 days the T24 cells were harvested using trypsin and the number of cells was counted.

A dose of 20 ng/ml aFGF was tested as chemoattractant for T24 using a modified Boyden chamber assay with 8 μm nuclepore filters (Keizer *et al.*, 1993). Incubation was for 4 h at 37°C in the absence of FCS. The number of cells migrated to the opposite side of the filter was counted.

RESULTS

Effects of growth factors on the lateral invasion of T24 cells

In explant cultures kept in standard medium containing 10% FCS the urothelium immediately began to expand as a cohesive sheet of urothelial cells after their attachment to the cyclopore membrane. On day 4, when the urothelial outgrowth covered more than 8.5% of the membrane, sheets of T24 cells were placed on the cyclopore membrane on each side of the outgrowth. On day 5 the standard medium was replaced or substituted for standard medium containing aFGF or EGF. Between day 7 and 9 the T24 cells and the growing urothelium came into contact. After day 12 the surface area of the bladder outgrowth reached a plateau, since the T24 cells became continuous with the primary outgrowth. The cultures were terminated on day 24 and immunostained with RCK 108. In overview sections it was apparent that in the control cultures (without the addition of growth factors to the standard medium) small numbers of individual T24 cells were infiltrating the primary bladder culture (Fig. 1*a*). In contrast, in cultures treated with aFGF the T24 cells massively invaded the periphery of the primary urothelium. On the other hand, in cultures treated with EGF much smaller numbers of T24 cells infiltrating the primary urothelium were observed (Fig. 1*b,c*). The number and lateral distance of individual infiltrating T24 cells in the EGF treated cultures was significantly decreased compared to the control or aFGF treated cultures ($p \leq 0.01$) (Fig. 2*a,b*). On cross sections it was shown that the T24 cells expanded by growing underneath the primary urothelium in the aFGF and control cultures. In the EGF treated cultures the T24 cells were found on top of the, in this case, thickened rim of the primary urothelial outgrowth, whereas small numbers of T24 cells also expanded underneath the bladder urothelium (data not shown).



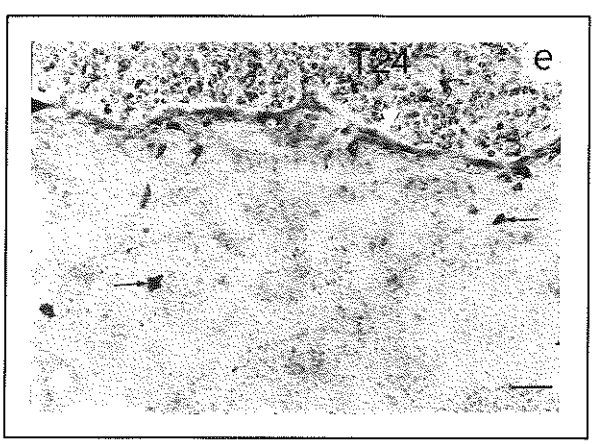


Figure 1 - Overview of cocultivation of the primary urothelium and T24 cells (×10). T24 cells are selectively stained with an antibody against cytokeratin 19. Nuclear counterstaining with haematoxylin. The marker (▲) indicates the border between T24 cells and the primary urothelial culture, arrows indicate individual infiltrating T24 cells in the primary urothelium. a) control culture, b) culture treated with aFGF, c) culture treated with EGF, d) culture grown on laminin and e) culture grown on collagen type IV. Scale bar = 100μm.

Effects of extracellular matrix components on the lateral invasion of T24 cells

The explants were placed on transparent membrane either pre-coated with Lam, FN, Col IV or Col I. On day 1 the explants started to expand on the membrane and on day 4 sheets of T24 cells were again inoculated next to the outgrowth. Also in these cases from day 12 on a further bladder outgrowth was inhibited by the T24 cells. The cultures were terminated on day 24 and immunostained with RCK 108. Invasion of T24 cells into the bladder outgrowth grown on Col IV, Col I and FN was only incidentally observed (Fig. 1*d*). The number and lateral distance of individual infiltrating T24 cells in the cultures grown on laminin was significantly increased compared to the cultures grown on Col I, Col IV and FN (Fig. 1*e* and 3*a,b*). On cross sections it was found that under all circumstances the T24 cells were infiltrating the urothelium outgrowth by expanding underneath the urothelium (data not shown).

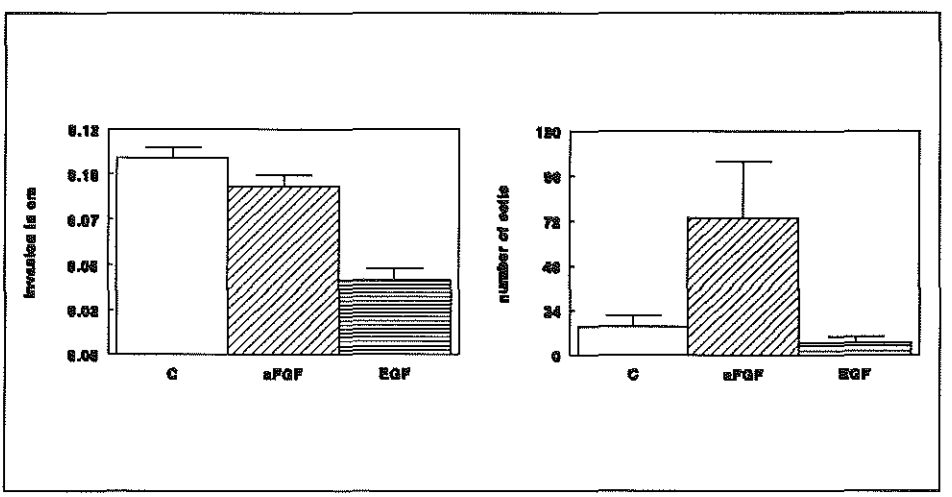


Figure 2 - Quantification of the lateral invasion of T24 cells in the explant cultures. The bars represent the mean ± SE of 3 independent experiments with 3 samples each. c = control culture. a) The distance of invasion of T24 cells in the primary culture in cm and b) Number of infiltrating T24 cells into the primary urothelium

Effects of growth factors and ECM components on migration and proliferation of T24 cells

EGF added to standard medium containing 10% FCS did not influence the ^{3}H thymidine incorporation compared to the control cultures. In aFGF treated cultures the ^{3}H thymidine incorporation decreased compared to the control cultures. Culturing of T24 cells on Lam, FN, Col I or Col IV substrate did not affect the ^{3}H thymidine incorporation (Fig.

4*a*). In the expansion assay on transparent membranes a significant decrease in expansion of the T24 cells was found on the FN coated membranes. In the other cases no significant changes in expansion of T24 cells was observed when compared to the 10% FCS control cultures (Fig. 4*b*). In order to study a potential effect of aFGF on the migration of T24 cells we used a modified Boyden chamber assay. in the chambers with aFGF 187.7 ± 22.9 cells were found on the opposite site of the filter compared to 185.6 ± 21.8 cells in control experiments. We conclude that aFGF does not affect the migration of T24 cells.

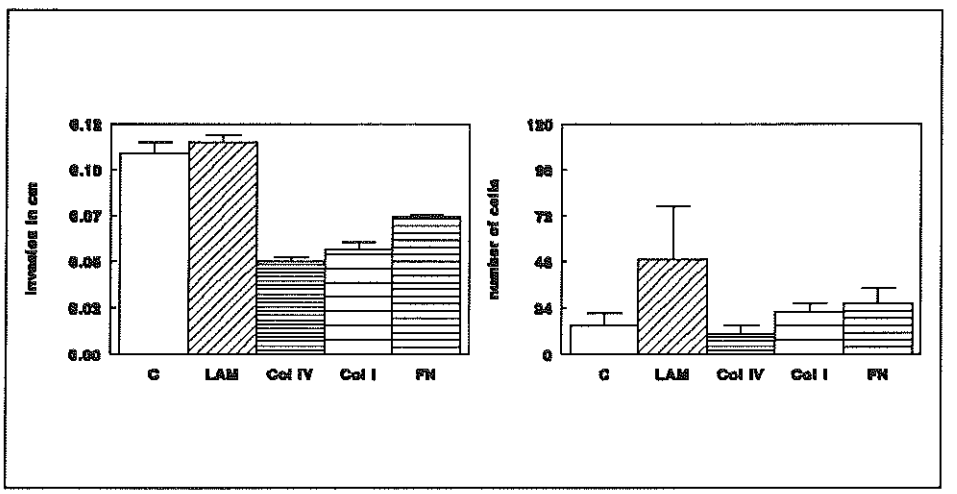


Figure 3 - Quantification of the lateral invasion of T24 cells in the explant cultures. The bars represent the mean ± SE of 3 independent experiments with 3 samples each. c = control culture. a) The distance of invasion of T24 cells in the primary culture in cm and b) Number of infiltrating T24 cells into the primary urothelium.

DISCUSSION

Carcinoma in situ represents the most common form of intra-epithelial neoplasia of the bladder and may be considered as the intra-epithelial propagation of tumour cells originating from an adjacent bladder carcinoma (Koss *et al.*, 1974; Mahadevia *et al.*, 1986). The pagetoid type of infiltration is another form of intra-epithelial spread of bladder carcinoma characterized by individual tumour cells infiltrating otherwise normal urothelium (Koss *et al.*, 1975). Patients with the latter infiltration pattern have the same progression and survival rates as patients with a carcinoma in situ (Orozoco *et al.*, 1993). The mechanisms underlying these two forms of intra-epithelial expansion have not been studied to a great extent, since in vitro models for intra-epithelial neoplasia are largely lacking.

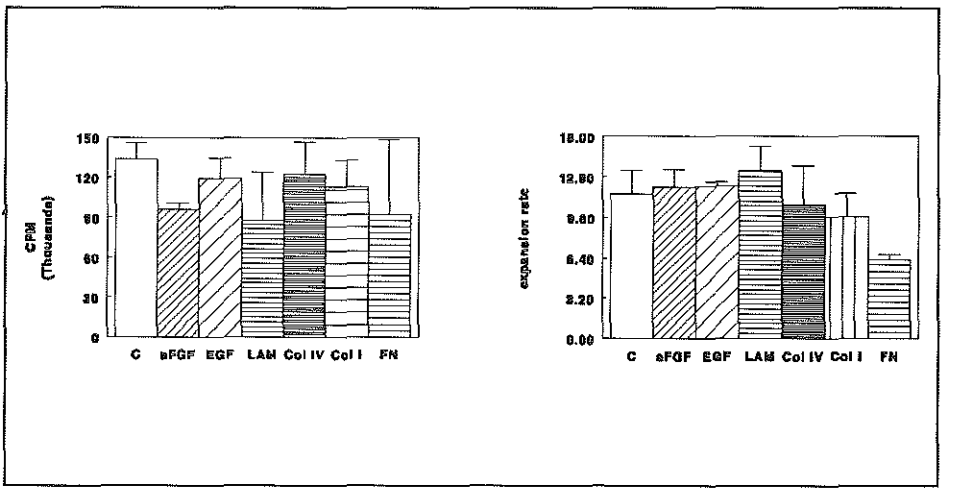


Figure 4 - Quantification of the expansion rate and proliferative activity of T24 cells. The bars represent the mean ± SD of 2 independent experiments with 3 samples each. c = control culture. a) ^{3}H thymidine incorporation in T24 cells and b) expansion rate of T24 cells

Murine bladder explants cultured on transparent cyclopore membranes expand rapidly and the final cultures closely mimic the in vivo situation even displaying maturation in umbrella cells. These cultures can easily be manipulated by adding growth factors to the standard medium (containing 10% FCS) or by pre-coating the transparent membranes with extracellular matrix components. Using this culture system we here describe an in vitro model that yields quantitative and qualitative data on the intra-epithelial spread of human T24 cells into primary organotypic cultures of mouse urothelium. In the cocultivation cultures the T24 cells could be selectively immunostained with the species-specific monoclonal antibody RCK 108 permitting their unequivocal identification (Rebel *et al.*, 1993).

In control cocultivation cultures a pagetoid infiltration pattern of the T24 cells into the urothelium, underneath the primary urothelium was found. We presume that an interaction of the T24 cells with the supportive membrane is required for their intra-epithelial infiltration. Our observation that ECM coating of the supportive membranes specifically influences the extent and number of infiltrating T24 cells would reinforce the above hypothesis.

The observed growth factor and ECM-mediated variations in number and distance of infiltrating T24 cells into the urothelium could be due to either a direct action of these components on T24 cells or indirectly via the primary urothelium. Migration of the T24 cells

was, however, not affected by exposure to growth factors or ECM components, except for FN. Proliferation of T24 cells was also not affected by ECM or EGF exposure. On the other hand, these growth factors and ECM components strongly influence the expansion rate, and level of differentiation and proliferative activity of the primary urothelial culture (Rebel *et al.*, 1994). Therefore we conclude that the difference in number of and distance covered by the infiltrating T24 cells in the primary urothelium must be ascribed to changes induced in the primary bladder culture, rather than a growth factor or ECM component mediated effect on the T24 cells. The decrease in distance and lower number of infiltrating T24 cells in cultures grown on FN, however, could be due to a direct migration inhibition of FN on T24 cells rather then on a FN mediated effect on the primary urothelium.

In a previous study we demonstrated that EGF, Col I, Col IV and FN exposed primary urothelial cultures have an increased number of cell layers when compared to the cultures grown on laminin (Rebel *et al.*, 1994). In the aFGF treated urothelium a decrease in nuclear density in the periphery of the urothelium, when cocultivated with T24 cells, was observed (Fig. 1*b*). Interestingly, in the cultures exposed to EGF, Col I, Col IV and FN, with more than one cell layer in the periphery, a decrease in distance and number of infiltrating T24 cells was observed compared to the urothelial cultures grown on laminin or treated with aFGF. Apparently, a reverse relation exists between cellular density of the primary urothelial culture and invasion of T24 cells.

Several studies have shown that growth factors can induce epithelial cells to produce ECM components. Fibronectin secretion by cornea epithelium, and collagen type IV secretion by lung epithelium is enhanced by EGF (Federspiel *et al.*, 1991; Schultz *et al.*, 1992). Drago *et al.* (1991) showed an enhanced laminin expression by neuroepithelial cells after stimulation with bFGF. T24 cells express $\alpha 3$, $\alpha 5$, $\alpha 6$, $\beta 1$ and $\beta 4$ integrin subunit enabling them to attach to and interact with different ECM substrates (Coplen *et al.*, 1991; Hogevorst *et al.*, 1993). Thus, induction of urothelial ECM synthesis by aFGF could also be a possible underlying mechanism by which T24 cells are permitted to adhere and migrate underneath the primary urothelial outgrowth.

In summary, the balance of transformed urothelial cells and (regenerating) urothelium is not only dependent on direct effects of growth factors and extracellular matrix components on transformed cells but, as this data implies, also on factors directly affecting the primary urothelium. Clinical relevance of this observation needs to be studied further.

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ACKNOWLEDGEMENTS

This study was supported by the Dutch Cancer Foundation (IKR 90-17). The authors would like to thank S. Erkens for the technical assistance and F.L. van der Panne for the photography.

CHAPTER 5

E-CADHERIN EXPRESSION DETERMINES THE MODE OF REPLACEMENT OF NORMAL UROTHELIUM BY HUMAN BLADDER CARCINOMA CELLS

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Cancer Research 54: 5488-5492; 1994

ABSTRACT

The high recurrence rate of human bladder cancer can be attributed to intra-epithelial expansion of tumour cells or shedding and subsequent implantation of tumour cells elsewhere in the bladder. E-cadherin is a calcium dependent cell-cell adhesion molecule and loss of E-cadherin by tumour cells is associated with increased tumour aggressiveness. Here we demonstrate that E-cadherin is also an important determinant of the mechanisms which are involved in the recurrence rate of bladder cancer. In a recently developed in vitro cocultivation model we studied the effect of E-cadherin expression on the intra-epithelial expansion pattern of 6 different human bladder carcinoma cell lines into primary murine urothelium. Bladder carcinoma cells lacking E-cadherin infiltrate into the primary urothelium as individual cells (pagetoid pattern). In contrast, a sharp demarcation is observed between E-cadherin positive bladder cancer cells and the primary urothelium (carcinoma in situ pattern). With the same model we demonstrate that only E-cadherin positive bladder carcinoma cell lines could attach to and colonize the intact primary urothelium. We hypothesize that it is the latter process that plays an important role in the high recurrence rate that is observed in some of the patients.

INTRODUCTION

Human bladder cancer is a common cancer in western society. Patients with superficial bladder cancer confined to the mucosa are considered to have a good prognosis. However, the major problem of patients with superficial bladder cancer is the high recurrence rate after local resection of the transitional cell carcinoma (1). These recurrent tumours probably develop from residual tumour cells and thus are of monoclonal origin (2, 3). Recurrence of bladder carcinomas could be due to the seeding of tumour cells on traumatized or even intact urothelium or by intra-epithelial expansion of the transformed cells from the original bladder carcinoma (4-6).

The cell-cell adhesion protein E-cadherin represents a cell surface glycoprotein which accounts for a Ca^{2+} dependent homotypic adhesion. It plays a role in organization and maintenance of tissue structure (7, 8). Lack of E-cadherin expression may lead to diminished cellular adhesion, resulting into invasion into connective tissue, and possibly to metastasis

(9). In some *in vitro* assays E-cadherin negative carcinoma cell lines but not E-cadherin positive cells were capable of invasion. Two well-differentiated E-cadherin positive human bladder carcinoma cell lines (RT 4 and RT 112) were not invasive into collagen matrices, while the E-cadherin negative human bladder carcinoma cell line EJ28 was able to invade the collagen matrices (10, 11). Invasive cell lines lost their ability to invade into chicken hart fragments after transfection with the E-cadherin cDNA.

Down-regulation of E-cadherin expression has been observed in a variety of infiltrating human cancers (12, 13). Moll et al. (14) noticed in infiltrating lobular breast carcinomas, characterized by dispersed invading neoplastic cells, a complete loss of E-cadherin expression. In prostate cancer the level of E-cadherin expression is inversely correlated with tumour differentiation and grade (15, 16). Thus, E-cadherin may act as an invasion suppressor molecule *in vivo* and *in vitro* (17).

Bringuier et al. (18) demonstrated that decreased E-cadherin expression correlated with a poor survival in patients with bladder cancer. In 76% of the invasive bladder tumours an abnormal or absent E-cadherin staining was found. The possible relationship between E-cadherin mediated cell-cell adhesion and the recurrence rate in bladder cancer has not been studied as yet. We investigated the role of E-cadherin in bladder carcinoma recurrences by addressing the following questions: 1) Does E-cadherin expression play a role in the intra-epithelial expansion of bladder carcinoma cells and 2) do bladder carcinoma cells attach and colonize the intact urothelium and, if so, is E-cadherin involved in this process? For these experiments we used a previously developed *in vitro* cocultivation system that permits the study of intra-epithelial expansion of bladder carcinoma cells into regenerating primary murine urothelium and the attachment of these cells to intact urothelium. In this model the primary murine urothelium shows multilayering and differentiation into umbrella cells which closely mimics the *in vivo* situation (19, 20). Primary urothelial cultures were cocultivated with different human bladder carcinoma cell lines with or without E-cadherin expression or with a murine bladder cell line lacking E-cadherin expression. Cell lines lacking E-cadherin expression infiltrate as individual cells into the primary urothelium. In contrast E-cadherin positive cells were able to attach to and replace the intact urothelium.

MATERIAL AND METHODS

Cell lines

Six human bladder carcinoma cell lines were used throughout these studies, namely T24, T24 2pn, J82, RT 112, SD/48 and Jon/53. The latter 4 cell lines were a generous gift of Dr J. A. Schalken, Urological Research Laboratory, University Hospital Nijmegen, The Netherlands (21, 22). T24 2pn is a subline derived from subcutaneous tumours in nude mice which developed from previously inoculated T24 cells after a latency of 17 weeks. One mouse bladder cell line was used: NUC-1 ßgal. NUC-1 ßgal was obtained by co-transfection of NUC-1 cells (23) with the plasmid pCMVß which contains the E-coli ß galactosidase gene (Clontech Laboratories, Palo Alto, USA) and a construct bearing the neomycin resistance gene (pKO) (24).

The cell lines were maintained in 10% foetal calf serum in a 1:1 mixture of Dulbecco's modified Eagle's medium and HAM's F10 supplemented with 10 μg/ml insulin, 5 nM selenite, 5 μg/ml transferrin and 50 nM 4-hydrocortisone-21 hemisuccinate. This medium is termed standard medium (19). Passage of confluent cell cultures was done by incubation with 0.05% trypsin and 0.02% EGTA for 3 min at 37°C after which the cells were resuspended in standard medium.

Bladder explant cultures on transparent membranes

Murine urinary bladders were obtained from female C3H/He mice (6-8 weeks old). The mucosa was stripped off from the underlying muscle layer and cut into two pieces, each half was placed on a transparent cyclopore membrane (Falcon cell-culture insert, Cyclopore membrane with a diameter of 25 mm; pore size 0.45μm). The lamina propria was in contact with the membrane. The standard medium in the lower and upper compartment was changed every other day (19, 25)

Intra-epithelial expansion assay

On day 5 after initiation of the primary urothelial culture the medium was removed from the upper compartment and sheets of human bladder carcinoma cells of 5 mm^2 were inoculated on opposite sites of the outgrowth of the bladder explant. The cell sheets were allowed to attach to the transparent membrane for two hours; subsequently the upper compartment was refilled with standard medium. The surface of the bladder outgrowth was monitored daily through a light microscope. From these overviews the surface area of the bladder outgrowth was drawn by hand every other day and the expansion of the outgrowth was calculated. The surface area as determined in mm^2 was related to the total surface area of the transparent membrane (i.e. 430 mm^2). When the outgrowth covered the whole membrane this was set as 100% outgrowth (19). After 3 weeks of cocultivation the cultures were terminated and immunohistochemically stained. Results were obtained from 3 independent experiments with 4 to 6 samples each.

Attachment assay

For this assay the transparent membranes were pre-coated with collagen type IV. When the outgrowth of the bladder covered 60% $\pm$ 5% of the membrane 1.10^5 human bladder carcinoma cells, or NUC-1 ßgal were added in standard medium in the upper compartment on top of the primary urothelium. The next day the cultures were either terminated or the medium in the upper compartment was changed with standard medium. After

termination the cultures were immunohistochemically stained.

Blocking of the E-cadherin cell-cell mediated adhesion was achieved with an E-cadherin antibody HECD-1 (British Biotech Products, Abingdon, UK). Blocking of E-cadherin mediated cell-cell adhesion with HECD-1 was previously described by Doki et al. (11). Cells were incubated with 0.05% trypsin with 0.02% EGTA for 5 min and resuspended in standard medium and cells were washed in PBS[2]. A total of 1.10^5 cells was incubated in 200 μl PBS containing different concentrations of HECD-1 and shaken for 30 min at 37°C. Subsequently 1.3 ml standard medium was added and the cells were plated in a 24 well dish. The concentration of HECD-1 by which cell-cell adhesion was blocked was determined in a pilot experiment by titration of the antibody and monitoring its effect on cell-cell attachment.

To study attachment to primary urothelium the HECD-1 incubated bladder carcinoma cells were placed on top of the primary urothelial outgrowth. On day one and day 4 the cultures were terminated. Results were obtained from 3 independent experiments with 4 to 6 samples each.

Motility assay

Bladder explants were placed on fibronectin coated coverslips (25 mm) and cultured for 4 days. T24 cells were placed on the primary urothelium and the coverslips were mounted in a Sykes-Moore chamber (Belco glass). Time-lapse video recording was performed over a period of 18 hours after adding the T24 cells on top of the primary murine urothelial outgrowth. The average speed of locomotion of the T24 cells was calculated as total track length divided by duration of the recording time as previously described (26).

Immunohistochemistry

The urothelial cultures were fixed with 70% ethanol for 24 hours. Pre-incubation was done with 10% normal goat serum for 15 min, and the membranes were incubated with the primary antibody RCK 108 in the experiments were human bladder carcinoma cells were used. Subsequently, an indirect conjugated peroxidase method was applied as described before (19). All membranes were counterstained with haematoxylin, dehydrated and mounted. Monoclonal antibody RCK 108 (kindly provided by Dr Ramaekers, University of Maastricht, NL) is specific for human cytokeratin 19 and lacks cross reactivity with murine epithelia. This feature permits the unequivocal demonstration of human bladder carcinoma cells in a xenogenic model (19). In experiments using the murine NUC-1 ßgal cell line the membranes were incubated, after fixation, with a PBS solution containing 1% 4-Cl-5-Br-3-indolyl-ß-galactosidase (x-gal), 5mM potassium ferrocyanide, 5 mM potassium ferricyanide and 2mM $MgCl_2$ for 10 hours. A blue precipitate identifies the presence of NUC-1 ßgal cells.

For the detection of E-cadherin in the human carcinoma cell lines the indirect conjugated peroxidase method was used, with a primary antibody against human E-cadherin (Organon technik NL). For the detection of E-cadherin in the primary murine urothelium an avidin biotin fluorescein isothiocyanate (FITC) was used. The primary antibody against murine E-cadherin designated DECMA-1 was obtained from Sigma (NL).

To evaluate the position of the immunostained human bladder carcinoma cells within the primary urothelium more accurately, strips of the immunostained cyclopore membranes were dipped in 5% gelatin, dried overnight in the air and embedded in paraffin. Cross sections perpendicular to the surface were made.

RESULTS

E-cadherin expression

In a first series of experiments we analyzed the E-cadherin expression and localization on the different cell lines. Using an antibody against E-cadherin we found that SD/48, RT 112 and Jon/53 cells express E-cadherin on the cell borders while the other cell lines lack E-cadherin expression entirely. The primary murine urothelial cultures express E-cadherin, particulary on the cell-cell borders of the umbrella cells (Fig. 1). The antibody HECD-1 against human E-cadherin is able to block cell-cell adhesion of SD/48 and RT 112 cells in a concentration of 40ng/ml for 1.10^5 SD/48 cells and 80ng/ml for 1.10^5 RT 112 cells. An antibody against prostate specific antigen (PSA) with the same Ig G subclass as HECD-1 was not able to block the cell-cell adhesion of these cells (Fig. 2a, b).

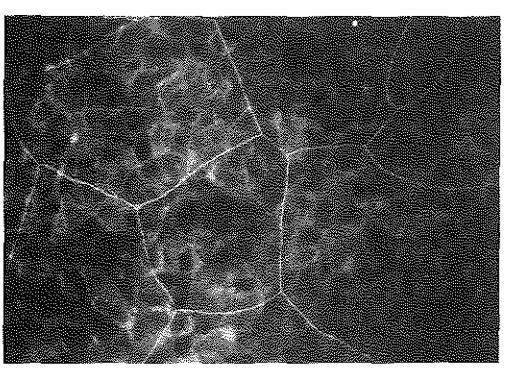

Figure 1- Overview of a primary urothelial culture stained with an antibody against E-cadherin (x 250). Scale bar = 50μm

Intra-epithelial expansion of human bladder carcinoma cells into the primary urothelium

Four days after initiation of the primary murine urothelial culture sheets of human bladder carcinoma cells were inoculated on both sides of the explant to study the intra-epithelial expansion of the tumour cells. At this time the surface of the primary urothelial culture exceeded 8.5% of the membrane. The edges of the primary urothelium were well demarcated throughout the experimental period. This permitted daily measurements of the surface covered by the expanding urothelial culture. Between day 7 and 9 the further expansion of the growing primary urothelium was inhibited when T24, T24 2pn, Jon/53, J82 or RT 112 cell sheets were inoculated. When SD/48 cells were inoculated the surface of the

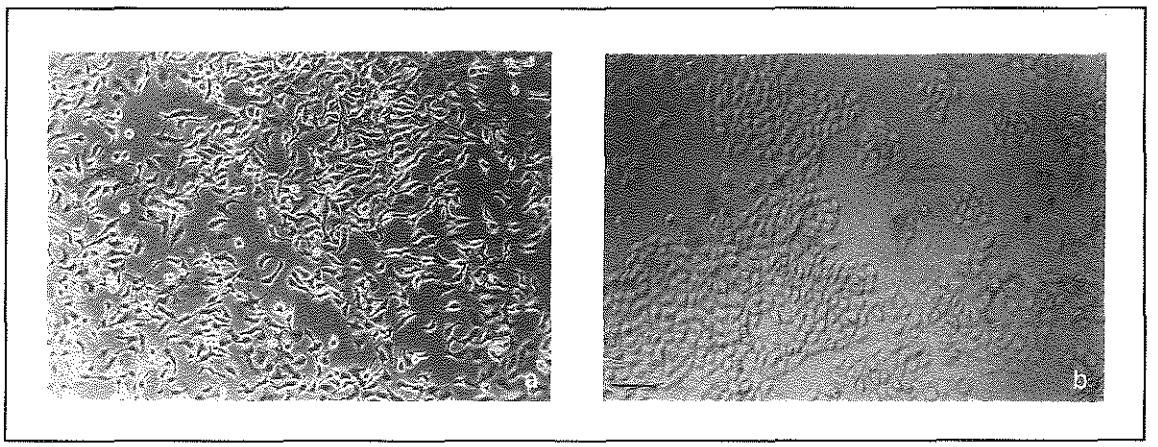

Figure 2- Phase-contrast micrograph of SD/48 cells (x 100) after incubation with a) 40 ng/ml HECD-1 (antibody against E-cadherin) and b) anti PSA (prostate specific antigen). Scale bar = 100μm

primary urothelium decreased in time after contiguity (data not shown). In overview sections stained with RCK 108 it was apparent that small numbers of individual E-cadherin negative human bladder carcinoma cells T24, T24 2pn and J82 were infiltrating the primary urothelial culture. In contrast, in cultures with the E-cadherin positive SD/48, RT 112 or Jon/53 cells a sharp demarcation between inoculated cells and primary urothelium was found with no individual infiltration by the human bladder carcinoma cells (Fig. 3a, b).

Attachment of bladder carcinoma cells on intact primary murine urothelium

To study the attachment of bladder carcinoma cells on top of the primary murine urothelium the medium of non confluent (60%) primary urothelial cultures was replaced with medium containing suspended bladder carcinoma cells. Subsequently, the cultures were terminated on day 1 or day 4, and stained with RCK 108 in the case of installation of human bladder carcinoma cells or stained for ß galactosidase activity in the case that NUC-1 ßgal was used. In all cases we observed that the E-cadherin negative cells (T24, T24 2pn, J82 and NUC-1 ßgal) did not attach to the primary urothelium after one or four days of culture. In contrast, the E-cadherin positive cells (SD/48, RT 112 and Jon/53) attached to the primary culture in one day and they developed clusters of cells which locally replaced the primary urothelium within 4 days (Fig. 4). Attachment and replacement by E-cadherin positive cell lines however, did only occur as dispersed clusters in a concentric peripheral zone of the

primary urothelium, were the E-cadherin positive primary urothelial umbrella cells are primary located.

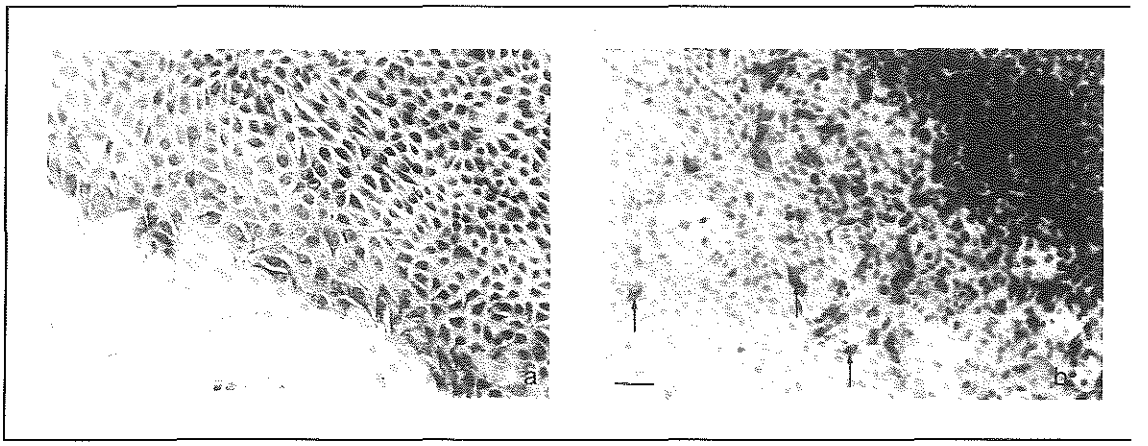

Figure 3- Overview of cocultivation of human bladder carcinoma cells which are inoculated next to the primary bladder outgrowth. Tumour cells are selectively stained with an antibody against human cytokeratin 19. Nuclear counterstaining was with haematoxylin (x 200) a) cocultivation with SD/48 b) cocultivation with T24. Arrows indicate individual infiltrating T24 cells in the primary urothelium. Scale bar = 50μm

Cross sections of the sites of attachment of E-cadherin positive cells to the primary urothelium clearly demonstrated that on day 1 the cells were on top of the primary urothelial outgrowth while on day 4 the bladder carcinoma cells had replaced the underlying primary urothelium.

In order to investigate how E-cadherin negative cells behave on top of the primary urothelium a timelapse video was recorded. T24 cells were selected and were added on top of the primary urothelium cultured on coverslips. A timelapse video recording the T24 cells revealed that T24 cells move (the speed is 49.4 μm/h $\pm$ 11.5 μm/h) along the borders between the umbrella cells but they are apparently unable to attach to these cells (Fig. 5a-d).

Blocking of cellular adhesion to primary urothelium with monoclonal antibody against E-cadherin

To investigate whether attachment of E-cadherin positive cells to intact primary urothelium is E-cadherin dependent suspensions of SD/48 or RT 112 cells were incubated with HECD-1 (anti E-cadherin) and added to the primary urothelial cultures. Incubation with HECD-1 prevented attachment of SD/48 cells and RT 112 cells to the primary urothelial

outgrowth after one day of culture. No clusters of attached cells were found. After 4 days of culture only a few HECD-1 incubated SD/48 cells were able to attach to the primary bladder culture but RT 112 cells did not attach to the primary urothelium. In control experiments, anti-PSA or PBS treated SD/48 or RT 112 cells did attach to the primary urothelium with an average of 91 cell clusters of SD/48 cells and 25 cell clusters of RT 112 cells per culture. T24 cells incubated with HECD-1 or anti PSA-antibody never attached to the primary urothelium.

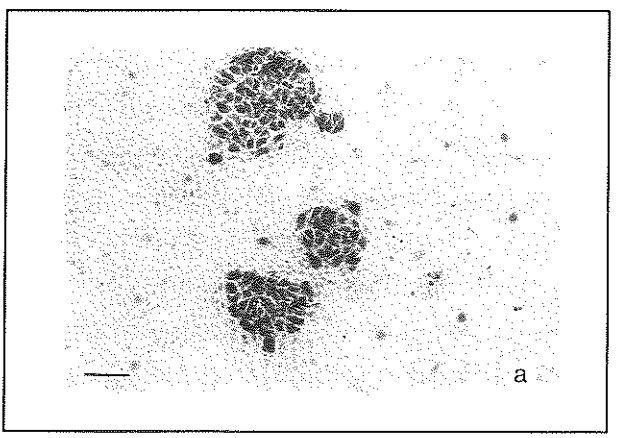

Figure 4- Adhesion of SD/48 to the primary urothelium. SD/48 cells are selectively stained with an antibody against human cytokeratin 19. a) overview of the SD/48 cells attached to the primary urothelium (x 200) Scale bar = 100μm .

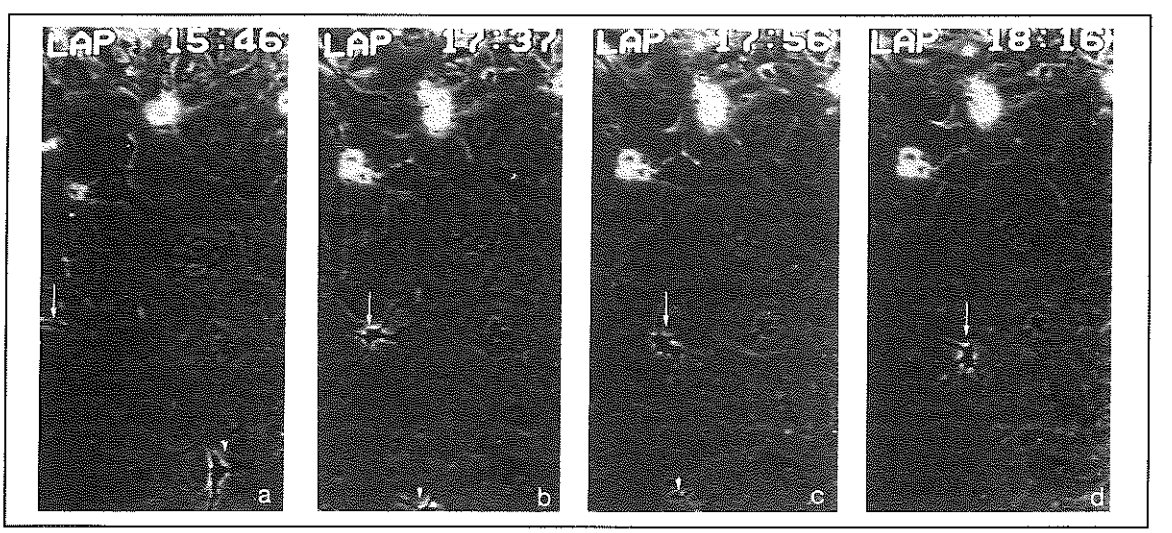

Figure 5- Photographs taken from a timelapse video showing migration of T24 cells on top of the primary urothelium. Arrows or arrowheads indicate the same T24 cell at different times after addition of the culture. a) 15 hrs and 46 min. b) 17 hrs and 37 min. c) 17 hrs and 56 min. d) 18 hrs and 16 min.

DISCUSSION

The high recurrence rate of bladder cancer is probably due to monoclonal expansion of remaining tumour cells after resection of the primary tumour. The most common form of intra-epithelial expansion in the bladder is the carcinoma in situ which may be considered as the intra-epithelial propagation of tumour cells originating from a adjacent bladder carcinoma (4). The pagetoid type of infiltration is another, less common, form of intra-epithelial expansion. This expansion is characterized by the penetration of individual transformed cells into otherwise morphologically normal stratified epithelium (6, 27, 28). The mechanism underlying these two forms of intra-epithelial spread have not been studied to a great extent, since *in vitro* models for intra-epithelial neoplasia are scant. Another important route which could lead to tumour recurrences, is the shedding of tumour cells with subsequent attachment to damaged or even intact urothelium and the formation of new carcinomas at distant sites.

In the cocultivation studies described in this paper we studied the influence of E-cadherin expression both 1) on the intra-epithelia expansion of the tumour cells, thereby mimicking the *in vivo* situation that would occur locally after removal of a primary tumour and 2) on the shedding and attachment process by applying a suspension of tumour cells to the primary urothelial culture. In both types of experiments we observed that expression of E-cadherin makes a marked difference on the behaviour of tumour cells with respect to the normal urothelium. From an adjacent monolayer of E-cadherin negative tumour cells, individual cells are able to invade the urothelial culture. This behaviour resembles the pagetoid type of infiltration discussed above. On the other hand we showed that all E-cadherin positive cell lines were unable to infiltrate the primary urothelial culture as individual cells. A sharp demarcation was observed between the primary urothelium and the E-cadherin positive tumour cells as is the case in carcinoma in situ. The level of inhibition of the urothelial outgrowth by the human bladder carcinoma cell lines was, however, not associated with E-cadherin expression or tumorigenicity (T24 compared to T24 2pn). The lack of homotypic cell-cell adhesion of E-cadherin negative cell lines seem to be a good explanation for our observations that E-cadherin negative bladder carcinoma cells could infiltrate as individual cells.

When we studied the behaviour of the tumour cells after their application on top of the primary urothelial culture, again there was a clear difference between E-cadherin positive

and negative cells. Cells expressing E-cadherin are apparently able to attach to the umbrella cells of the explant culture. These cells express E-cadherin themselves and, taking into account that an antibody against E-cadherin was able to prevent attachment, it is likely that the tumour cells use these interactions to stably attach to the urothelium. In contrast, none of the cell lines lacking E-cadherin were able to attach to the primary urothelium. Indeed, E-cadherin negative T24 cells were found to move across the urothelial culture but could not attach.

Several other groups have investigated the implantation of transformed cells in *in vivo* and *in vitro* models. Those studies that were performed using partially traumatized urothelium suggested that implantation of tumour cells occurs mostly in the injured area of the bladder (29-32). Soloway and Masters (5) also demonstrated that tumour cells could attach to intact urothelium *in vivo*, however, this occurred considerably less frequently when compared to traumatized urothelium. In our study we show that attachment to intact urothelium *in vitro* is a frequent event and completely dependent on E-cadherin expression. Similar findings have been reported by Tang et al. (33) who demonstrated that the adhesion of human melanocytes to a keratinocytes monolayer *in vitro* is E-cadherin mediated. If E-cadherin of the melanocytes is blocked with an antibody or if E-cadherin is degraded by trypsin the adhesion of these melanocytes to the keratinocyte monolayer is reduced. They also showed that when the E-cadherin expression was lost in one of the cell types no adhesion was found.

When we combine our *in vitro* results with the *in vivo* observations the data suggests that the two forms of intra-epithelial expansion fundamentally differ: E-cadherin positive bladder tumours have a carcinoma in situ like expansion while E-cadherin negative tumours expand as a pagetoid infiltration pattern. E-cadherin negative bladder tumours are found to be the more aggressive ones (18, 34) so it is likely that the individual infiltrative E-cadherin negative cells (which infiltrate underneath the primary urothelium [19]) could finally infiltrate the lamina propria to form tumours with a more aggressive biological behaviour.

The process of shedding of E-cadherin positive tumour cells and their attachment on intact urothelium leading to new tumours elsewhere in the bladder probably occurs rather frequently. Therefore we hypothesize that this E-cadherin dependent mechanism accounts for a large proportion of bladder tumour recurrences and this process could also explain the multifocality of bladder tumours observed at first diagnosis.

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CHAPTER 6

ACTIVELY PROLIFERATING UROTHELIUM INHIBITS IMPLANTATION AND GROWTH OF T24 BLADDER TUMOUR CELLS IN TRAUMATIZED UROTHELIAL CULTURES

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submitted (1994)

ABSTRACT

A major problem for patients with bladder cancer is the high recurrence rate after local resection of the tumour. The impact of the regenerative activity of primary urothelium on tumour cell implantation was investigated in a cocultivation model. Murine bladder explants on transparent membranes mimic the in vivo situation with respect to multilayering and maturation. Human T24 bladder carcinoma cells did not attach to intact primary urothelium even when the maturation of primary urothelium was modulated by exposure to growth factors and extracellular matrix components. After denudation of small areas within confluent primary cultures the lesions were covered by urothelium within 16 hours. T24 cells were found to attach exclusively to the injured areas and fill the lesion. Exposure of the primary urothelium to epidermal growth factor enhanced the proliferation of the primary urothelium and reduced the area covered by inoculated T24 cells conssiderably. These data suggest that the recurrence rate of some transitional cell carcinomas after surgical manipulation may be reduced by stimulating the proliferative activity of normal urothelial cells.

INTRODUCTION

Human bladder cancer is a common cancer in western society. Over 70% of these tumours are superficial and only 15% progress to invasive tumours.[1] The major problem of patients with superficial bladder cancer is the high recurrence rate after local resection of the transitional cell carcinoma (TCC).[2,3] On the other hand, the regeneration rate of damaged urothelium itself is very high when compared to the slow cell turn over of the urothelium under normal circumstances.[4-7] Originally, it was thought that recurrent tumours represented de novo tumours. Now, it has been demonstrated that these recurrent tumours are of monoclonal origin, implying that these tumours are derived from residual tumour cells.[8,9]

Clinical and experimental data indicate that a substantial proportion of bladder carcinoma recurrences could be the consequence of shedding and subsequent reattachment of tumour cells to traumatized areas in the urothelium. It was demonstrated that when the bladders of rats or mice were injured and tumour cells were inoculated in the bladder, successful implantation of tumour cells occurred mostly in the traumatized regions in the

urothelium.[10-12] Previously, we demonstrated that only E-cadherin positive bladder carcinoma cells were able to attach and colonize intact urothelium and that the potentially more invasive E-cadherin negative bladder carcinoma cells could not attach to intact urothelium.[13]

See et al.[14] demonstrated that the size of the tumour after inoculation of tumour cells in injured rat bladders is linearly related to the original surface of the injury in the urothelium. Because normal bladder epithelium apparently also has a considerable growth potential[4-7] we intended to investigate whether outgrowth of implanted E-cadherin negative tumour cells on traumatized urothelium could be modulated by influencing the regeneration rate of the normal urothelium.

For this purpose, we used a recently developed in vitro cocultivation model that permits the study of tumour cell growth in traumatized urothelium. In explant cultures the primary murine urothelium shows multilayering and differentiation into umbrella cells which closely mimic the in vivo situation.[15] In this in vitro model the expansion rate was shown to be enhanced by addition of epidermal growth factor (EGF).[16] In this paper we show that the human T24 bladder carcinoma cells are not able to attach on intact murine urothelium even if the maturation and differentiation of this urothelium is influenced by external factors. The T24 cells exclusively attach to and colonize the denuded areas in the confluent primary urothelial cultures. Addition of EGF prior to the injury can stimulate the regeneration of the primary urothelial cells to such an extent that only a fraction of the original lesion is taken up by tumour cells.

MATERIAL AND METHODS

Bladder explant cultures on transparent membranes.

Murine urinary bladders were obtained from female C3H/He mice (6-8 weeks old). The mucosa was stripped off from the underlying muscle layer and cut into two pieces, each half was placed on a collagen type IV pre-coated transparent cyclopore membrane (Falcon cell-culture insert, Cyclopore membrane with a diameter of 25 mm; pore size 0.45μm). The lamina propria was in contact with the membrane. The primary urothelium was maintained in 10% foetal calf serum in a 1:1 mixture of Dulbecco's modified Eagle's medium and HAM's F10 supplemented with 10 μg/ml insulin, 5 nM selenite, 5 μg/ml transferrin and 50 nM 4-hydrocortisone-21 hemisuccinate. This medium is termed standard medium.[15] The standard medium in the lower and upper compartment was changed every other day.

In some experiments, 20 ng/ml EGF (Sigma, St. Louis, USA) was added to the standard medium beginning at day 4 (as indicated in the text). This EGF supplemented medium was changed every other day.

Inoculation of T24 cells on injured urothelium

When the outgrowth of the bladder covered 60% ± 5% of the membrane the primary urothelium was injured with a pair of tweezers: within one primary urothelial culture 8 small areas were denuded with an approximate size of 1 mm^2 as judged from microscopic measurements. Four injuries were made in a concentric zone in the periphery and four injuries were made in the more central part of the culture. The urothelium was washed twice with PBS and a single cell suspension of 1.10^5 human T24 bladder carcinoma cells was added in standard medium (in some experiments EGF supplemented medium) to the upper compartment on top of the primary urothelium. The next day the cultures were either terminated or the medium in the upper compartment was changed with standard medium or EGF supplemented medium. After termination the cultures were immunohistochemically stained.

Immunohistochemistry

The urothelial cultures were fixed with 70% ethanol for 24 hours. Pre-incubation of the membranes was done with 10% normal goat serum for 15 min, followed by an incubation with the primary antibody RCK 108. Subsequently, an indirect conjugated peroxidase method was applied as described before.[15] All membranes were counterstained with haematoxylin, dehydrated and mounted. Monoclonal antibody RCK 108 (kindly provided by Dr Ramaekers, University of Maastricht, NL) is specific for human cytokeratin 19 and lacks cross reactivity with murine epithelia. This feature permits the unequivocal demonstration of human T24 bladder carcinoma cells in this xenogenic model.[15]

Quantification of the tumour size in the urothelium

Quantification of the area covered by T24 tumour cells in the primary urothelium culture was done using an image analyzer (IBAS 2000 Zeiss Kontron, Oberkochen, FRG). The area and expansion of attached T24 cells was visualized with a Hitachi CCTV camera and analyzed with the supplied Kontron IBAS1 version 4.4 software program. Quantification was done on the 4 central and 4 peripheral injuries on day 1 and 5 after inoculation of the T24 cells. Data are expressed as the mean ± SEM of three independent experiments with three urothelial cultures each.

RESULTS

Inoculation of T24 cells on intact urothelium

E-cadherin negative cells are not able to attach to intact normal murine urothelium, while E-cadherin positive cells can attach and colonize this urothelium.[13] To investigate whether the E-cadherin negative T24 bladder carcinoma cells are able to attach to modulated intact urothelium a single cell suspension of the human bladder carcinoma T24 cells was added to primary urothelial cultures exposed to a variety of different growth factors or extracellular matrix components. Previously, we demonstrated that EGF, acidic fibroblast growth factor, laminin and collagen type IV could influence the multilayering and

differentiation into umbrella cells of the primary urothelial culture.[16] When a suspension of T24 cells was added to urothelial cultures exposed to one of the above mentioned growth factors or ECM components the T24 cells were not able to attach to any of these urothelial cultures as judged from the lack of staining with the species specific antibody against cytokeratin 19. More in particular, no attachment was found in cultures lacking a superficial cell layer with the morphology of umbrella cells and no attachment was found in cultures with only one (flattened) cell layer.

Inoculation of T24 cells on traumatized urothelium

To investigate if T24 cells are able to attach and grow in a damaged area of the urothelium, injuries were made in the primary urothelial culture. The primary urothelium was grown on collagen IV pre-coated membranes in order to mimic the in vivo situation as close as possible.[16] In the central part of these cultures a large proportion of the urothelial cells is small-sized and display proliferative activity while in the more peripheral concentric zones of the culture the cellularity is less and cells of the top layer have the morphology of umbrella cells. In this latter area of the culture hardly any proliferative cell is found.[16]

In the injured spots in the centre and periphery of the primary urothelial culture all cell layers of the urothelium were removed without damaging the cyclopore membrane (Fig 1a). This urothelial lesion recovers within 16 hours (Fig 1b).

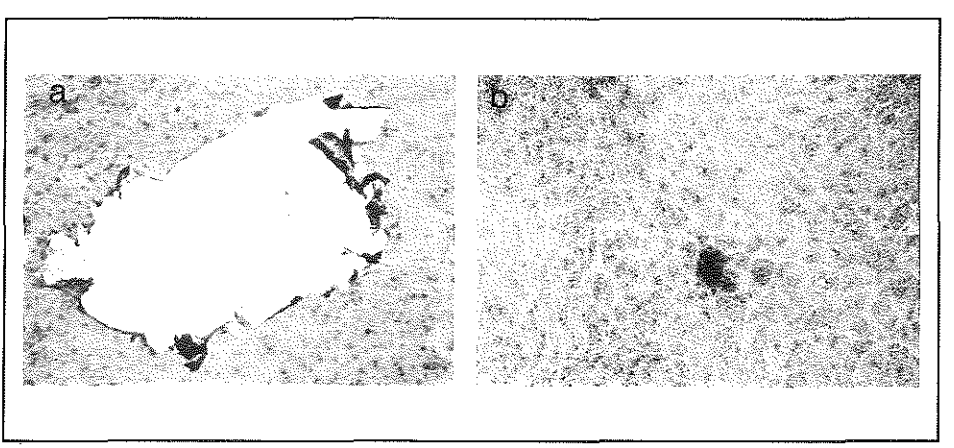

Figure 1- Overview of primary urothelium. The cultures were immunohistologically stained with an antibody specific for human cytokeratin 19 and counterstaind with haematoxylin. Magnification x 50 a) Overview of the injury in the urothelium b) overview of the urothelium one day after the injury was made

After inoculation of T24 cells to injured cultures these cells exclusively attached to the lesions. After 24 hours the lesion was filled up with T24 cells and regenerating urothelium. As demonstrated in Fig 2a the (RCK 108 positive) T24 cells were able to infiltrate the surrounding regenerating urothelium as individual cells. Morphometric studies revealed that after 5 days of cocultivation the area occupied by T24 cells increased in the periphery of the primary urothelial cultures (Fig 3). In the central, more rapidly proliferating, zone the area populated by T24 cells decreased somewhat after 5 days of culture. Comparison of the lesions in the peripheral and more central zones of the culture showed that the size of T24 occupied areas was smaller in the central zone located lesions (Fig 3).

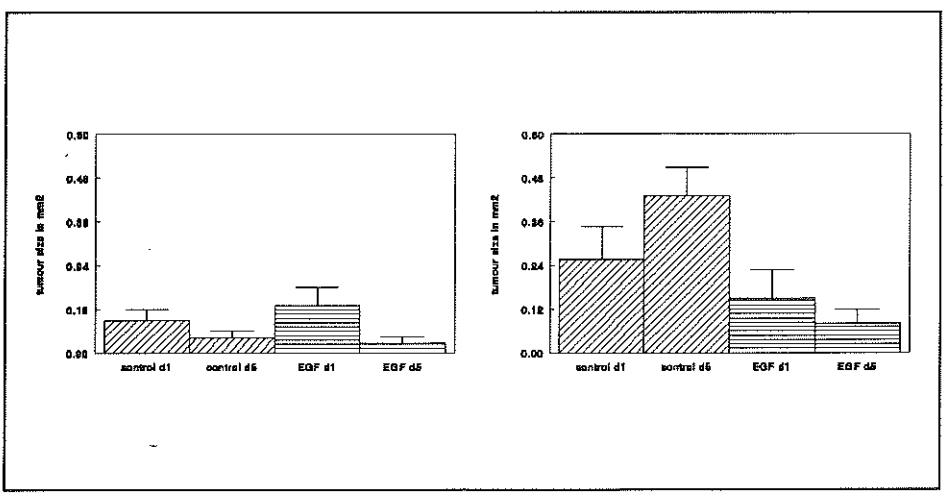

Figure 3- Quantification of the occupied area of T24 cells in the lesion of the urothelium. a) the middle of the urothelial culture b) the peripheral zone of the urothelial culture. Data are expressed as the mean ± SEM of three independent experiments with three samples each

Inoculation of T24 cells on traumatized urothelium in combination with EGF exposure

To the primary urothelial cultures EGF was added 5 days before the injuries were made. A suspension of T24 cells was added to the injured urothelial cultures and the cultures were supplied with EGF supplemented standard medium. Again, the T24 cells were only able to attach to the lesions. After 24 hours the lesions were occupied by T24 cells and regenerating urothelial cells. The T24 cells intervened as individual cells within the regenerating urothelium. Fig 2 and 3 show that particularly in the peripheral zone of the

urothelial cultures exposure to EGF results to a decrease in T24 populated area compared to non-treated cultures in the same experiment. Figure 3 shows that in the EGF treated cultures the area populated by T24 cells was decreased on day 5 compared to day 1.

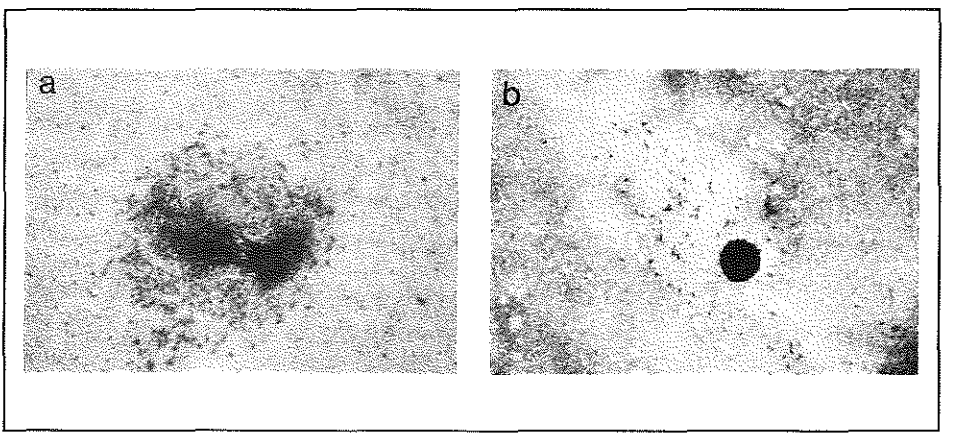

Figure 2- Overview of an injured urothelial culture cocultivated with T24 cells for 5 days. Cultures are stained with RCK 108 an antibody specific for human cytokeratin 19. The stained cells are T24 cells. Magnification x 50. a) control culture b) culture exposed to EGF

DISCUSSION

The high recurrence rate of bladder cancer after local resection of the primary tumour can be ascribed in a proportion of the cases to implantation of the remaining tumour cells on the traumatized urothelium. The therapy for superficial bladder cancer involves transurethral resection (TUR), causing traumatization of the urothelium and a release of tumour cells into the intravesical fluid. Patient studies showed that these cells are able to attach to the urothelium at distant sites relative to the primary carcinoma.[17,18] Soloway et al.[19] noted in an in vivo model that tumour cells implanted with a higher frequency on traumatized urothelium when compared to intact murine urothelium. Here we demonstrate that in our in vitro model the E-cadherin negative T24 cells are not able to attach to intact urothelium. In a previous study we demonstrated that E-cadherin positive cells are able to attach to intact urothelium and that this attachment is E-cadherin mediated.[13]

In order to study if attachment of the E-cadherin negative T24 cells to intact urothelium can be induced by changing the differentiation status of the primary urothelium,

the cultures were exposed to extracellular matrix components or growth factors. When urothelial proliferation was stimulated with EGF the T24 cells still did not attach although in these EGF treated urothelial cultures a superficial layer with the morphology of umbrella cells is not formed. Also in the situation where the urothelium largely consists of only one flattened cell layer, which is the case when the membrane is pre-coated with laminin,[16] the T24 cells were unable to attach to the intact primary urothelium. Thus, we conclude that T24 cells are unable to attach on intact urothelium, regardless its differentiation status.

T24 cells attach to and colonize the traumatized area of the primary urothelium. This is comparable with in vivo studies in which tumour cells attached to the injured urothelium.[10,12,19] Several in vivo studies indicate that intravesical chemotherapy might kill the residual tumour cells or prevent attachment of these cells to the urothelium.[20,21] Pan et al.[21] showed that installation of the chemotherapeutic agent thiotepa one hour after inoculation of tumour cells in a cauterized mouse bladder, decreases the implantation rate of the tumour cells. They suggested that the molecular composition of the urothelial surface is changed and that the tumours cells were inhibited in their attachment. No experimental studies, however, were done to investigate if an enhanced regeneration rate of the urothelium after TUR would influence implantation and colonization by tumour cells.

In non-EGF-treated cultures we noted that in the central parts the size of the areas populated with T24 cells is smaller compared to their size in the periphery of the culture. Also the area covered with T24 cells decreased somewhat in time in the central part while their sizes increased in time in the periphery of the urothelial culture. Thus, the difference in urothelial differentiation and cellularity in the central zones of the culture compared to the periphery could influence the repopulation of denuded areas by T24 cells. The small cells in the central part of the culture, with the morphology of basal cells, reflected by the higher cellularity and higher proliferative activity may counteract the expansion of T24 cells. This stands in contrast to the injured areas in the periphery of the primary urothelium where the T24 cells can expand in time.

EGF enhances the regeneration rate of the primary urothelium by stimulating their proliferation. In these EGF treated urothelial cultures the proliferative small cells are found throughout the whole culture and no umbrella cells are found.[16] In the peripheral and in the central part of these EGF treated cultures the area occupied by T24 cells decreases in time. Thus, here also the proliferating small urothelial cells are able to decrease the size of the area

covered with T24 cells.

These in vitro data support the hypothesis that enhancement of regenerative capacity of the urothelium by an increase in proliferation counteracts implantation and colonization of bladder carcinoma cells in urothelium. In vivo studies with a larger number of bladder carcinoma cell lines are required to demonstrate the potential benefit of this approach for therapeutical purposes.

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CHAPTER 7

SUMMARY AND CONCLUDING REMARKS

SAMENVATTING

SUMMARY

The major problem in the management of patients with superficial bladder cancer (Ta/T1) is the high recurrence rate after local resection of the primary tumour. There are two potential pathways for these tumour recurrences: 1) the intra-epithelial lateral infiltration of tumour cells from the primary tumour and 2) the shedding of tumour cells from the primary tumour and implantation of the shed cells randomly in the bladder on either intact or traumatized urothelium. To study either of these pathways for tumour recurrence we designed an *in vitro* model in which murine bladder explants were allowed to grow on transparent cyclopore membranes. The outgrowth of the bladder explant closely mimics the *in vivo* murine urothelium since multilayering and differentiation into umbrella cells occur. This primary urothelium was cocultivated with bladder carcinoma cells in order to examine lateral infiltration of tumour cells into primary urothelium. Alternatively, tumour cells were inoculated on intact or traumatized urothelium to study their attachment and implantation.

The experiments described in chapter 2 show that, when the primary murine urothelial cells are cocultivated with a non tumorigenic mouse bladder cell line (NUC-5) on the same membrane, the primary urothelium is able to displace the NUC-5 cell line. However, after introduction of the transforming DNA region from polyoma virus in this cell line, the now tumorigenic NUC-5 Py cell line was able to inhibit the outgrowth of the primary urothelium. In addition the transformed cells were found to grow underneath the primary urothelial culture. The human bladder carcinoma cell line T24 is also able to grow underneath the primary urothelial culture and inhibit the growth of the primary urothelium. The T24 cells penetrate into the urothelium as individual cells. In this xenogenic cocultivation experiment the T24 cells were identified by immunohistochemistry using a species-specific anti-cytokeratin 19 antibody. The pattern of infiltration of the T24 cells resembles the pagetoid infiltration pattern found in some patients with bladder carcinomas. These observation indicates that this cocultivation model is suitable to study the mechanisms of intra-epithelial lateral expansion of tumour cells.

Subsequently, the mechanisms of intra-epithelial lateral infiltration of T24 cells into normal murine urothelium was studied. We assumed that the degree of intra-epithelial lateral

infiltration of tumour cells into the primary urothelium might be dependent on the regenerating capacity of the primary urothelial cell culture and on the intrinsic characteristics of the transformed cells. Therefore, an alteration in the balance between the expansion of the primary urothelium and the transformed bladder cells might influence the extent of intra-epithelial lateral infiltration of the tumour cells. We first investigated how growth of the primary urothelium might be modulated by a variety of growth factors and extracellular matrix components.

The experiments in chapter 3 demonstrate that the expansion rate of the primary urothelium is influenced by growth factors and extracellular matrix components. EGF, aFGF and laminin were found to enhance the expansion rate of the primary urothelium in different ways. EGF primarily enhanced cell proliferation in the whole primary bladder culture also the surface area covered by the urothelial culture increased in the presence of aFGF, which is probably due to enhanced migration of the urothelial cells rather than increased proliferation. In the cultures treated with EGF no superficial cells with the morphology of umbrella cells were found. Urothelial cultures grown on laminin consist only of one cell layer composed of very flat and large cells. The observed increase in expansion of primary urothelium grown on laminin must therefore be ascribed to the flattening of the epithelial cells. It can be concluded that the expansion rate, differentiation and multilayering of primary murine urothelial cells are modulated by growth factors and ECM components.

The observed effects of growth factors and ECM components prompted us to study their effect on intra-epithelial lateral infiltration of T24 tumour cells into the urothelium. These experiments are described in chapter 4. Addition of EGF reduced the extent of intra-epithelial infiltration of T24 cells into the primary urothelium. Both the number and distance covered by T24 cells infiltrating into the urothelium was reduced. If the primary urothelium was grown on laminin or treated with aFGF the number of T24 cells infiltrating into the urothelial cell culture was enhanced in comparison to the non-treated urothelium. Remarkably, none of the tested growth factors or ECM components, influenced the migration and proliferation of the T24 cells. As described in chapter 3 growth factors and ECM components do modulate morphology and differentiation of the primary urothelium. Thus the difference in intra-epithelial expansion of the T24 cells into the urothelium must be due to effects mediated by the primary urothelial culture rather than a direct effect of the growth

factors and ECM proteins on the tumour cells. By light microscopical examination it became apparent that a reverse correlation exists between nuclear density in the primary urothelial cell culture and the distance and number of infiltrating individual T24 cells.

In the experiments described in chapter 5 we compared E-cadherin negative and positive bladder carcinoma cell lines with regard to their lateral expansion pattern. E-cadherin negative cell lines like T24 infiltrate the primary urothelium as individual cells. E-cadherin positive bladder carcinoma cells on the other hand do not infiltrate as individual cells but form cohesive sheets of tumour cells which inhibit the outgrowth of the primary urothelium. Thus E-cadherin negative bladder carcinoma cell lines infiltrate with a pagetoid infiltration pattern while E-cadherin positive cells have an expansion pattern characteristic of a carcinoma in situ.

In the experiments described in chapters 5 and 6 we studied shedding from and attachment of tumour cells on intact and damaged urothelium. We noticed that only E-cadherin positive cells could attach to intact murine urothelium. In contrast, E-cadherin negative cells were not able to attach to intact urothelium even when the urothelium had a changed morphology due to exposure to various ECM components or growth factors. Apparently, the differentiation of primary urothelium did not affect the adhesion of E-cadherin negative tumour cells. Since the attachment of E-cadherin positive tumour cells to the primary urothelium can be specifically blocked with an antibody against E-cadherin we concluded that the attachment of E-cadherin positive bladder carcinoma cells on intact urothelium is E-cadherin mediated. Finally we found that the E-cadherin negative T24 cells can attach to small lesions that were introduced in the confluent cultures of intact urothelium. These T24 cells then infiltrate the surrounding urothelium. The infiltration into the regenerating urothelium and the area covered by T24 cells in these denuded areas could be reduced by addition of EGF before and after the injuries in the urothelium were made.

In conclusion:

1) This in vitro cocultivation model is suitable to study both potential pathways that might be involved in the recurrence of superficial bladder cancer.
2) Growth factors and ECM components modulate pagetoid infiltration of E-cadherin negative T24 human bladder carcinoma cells indirectly by their interaction with the

primary urothelium rather than directly with the tumour cells.

3) E-cadherin expression modulate the pattern of lateral expansion.
4) Only E-cadherin positive tumour cell can attach to intact urothelium.
5) Growth factors influence the size of the area covered by T24 tumour cells in traumatized areas of the bladder.

Further studies should be designed to examine therapeutical possibilities, based on the findings in this study. Installation of EGF in the bladder before TUR is performed could reduce the recurrence rate in vivo. Our in vitro results suggest that addition of EGF can reduce the intra-epithelial lateral infiltration of E-cadherin negative tumour cells and enhances the regeneration rate of the primary urothelium. Recurrences of E-cadherin positive bladder carcinoma cells by their implantation on intact urothelium might be prevented by elimination of Ca^{2+} with Ca^{2+}-binders. Alternatively E-cadherin specific antagonists may be employed to this purpose.

SAMENVATTING

Het grootste probleem bij de behandeling van patiënten met blaaskanker (Ta/T1) is de hoge kans op een recidief na de verwijdering van de primaire tumor. Er zijn twee mogelijke mechanismen, die het optreden van de recidief tumoren kunnen verklaren: 1) de intra-epitheliale laterale infiltratie van tumor cellen vanuit de primaire tumor en 2) het loslaten van tumor cellen van de primaire tumor en de implantatie van deze tumor cellen op intact of getraumatiseerd urotheel. Om beide mechanismen ter verklaring van recidief-vorming van blaastumoren te bestuderen hebben wij een in vitro model ontworpen. Een halve muizeblaas krijgt de mogelijkheid om op een transparante cyclopore membraan uit te groeien. Deze in vitro uitgroei van blaasurotheel toont sterke morfologische overeenkomst met het urotheel in vivo. Er worden meerdere lagen epitheel gevormd en er is sprake van differentiatie tot paraplu cellen. Dergelijk in vitro groeiend primair urotheel werd samengebracht met blaastumor-cellen om de laterale infiltratie van de tumor cellen in het primaire urotheel te bestuderen. Om de implantatie en hechting van tumor cellen te bestuderen werden tumor cellen op getraumatiseerd of intact primair urotheel geënt.

De experimenten die in hoofdstuk 2 worden beschreven, laten de resultaten zien van de cocultivatie van primair muizeurotheel met een niet tumorigene muizeblaas cellijn en verschillende tumorigene cellijnen. Het primaire urotheel blijkt in staat om de niet tumorigene NUC-5 cellijn te verdringen. Na introductie van het transformerende DNA gedeelte van het polyoma virus in deze cellijn, is de nu tumorigene cellijn NUC-5 Py in staat om de uitgroei van het primaire blaas-epitheel te remmen. De tumorigene NUC-5 Py cellen breiden zich uit onder het primaire urotheel door. De menselijke blaaskanker-cellijn T24 is ook in staat om onder het primaire muize-urotheel door te groeien en de uitgroei van dit primaire urotheel te remmen. De T24 cellijn infiltreert het primaire urotheel als individuele cellen. In dit xenogene cocultivatie model kunnen de T24 cellen immunohistochemisch gekleurd worden met een diersoort-specifiek antilichaam gericht tegen cytokeratine 19. Het uitbreidingspatroon van de T24 cellen komt overeen met het pagetoide infiltratie patroon, dat is gevonden bij sommige patiënten met blaaskanker. Deze observaties leiden tot de conclusie dat dit cocultivatie model gebruikt kan worden om het mechanisme van de intra-epitheliale laterale expansie te bestuderen.

In hoofdstuk 4 wordt het mechanisme van de intra-epitheliale laterale infiltratie van T24 cellen in normaal muizeurotheel beschreven. Er wordt verondersteld dat de mate van intra-epitheliale infiltratie van tumorcellen in het primaire urotheel afhankelijk zou kunnen zijn van zowel de regeneratie capaciteit van het primaire urotheel als de karakteristieke eigenschappen van de getransformeerde cellen. Een verandering in de balans tussen de expansie van tumorcellen en het primaire urotheel zou zo de mate van laterale infiltratie van tumorcellen kunnen beïnvloeden. Eerst is onderzocht of de groei van het primaire urotheel gemoduleerd kon worden door verschillende groeifactoren en extra-cellulaire matrix componenten (ECM).

De experimenten in hoofdstuk 3 tonen aan dat de uitgroei-snelheid van het primaire muizeurotheel wordt beïnvloed door groeifactoren en extracellulaire matrix componenten. EGF, aFGF en laminine verhogen de uitgroeisnelheid van het primaire urotheel op verschillende manieren. EGF verhoogd de proliferatie in de gehele urotheelkweek. In deze aan EGF blootgestelde kweken van muizeurotheel worden overigens ook geen oppervlakte cellen gevonden met de typische morfologie van paraplu cellen.

In aanwezigheid van aFGF blijkt de uitgroei van het urotheel ook te zijn verhoogd dit wordt waarschijnlijk veroorzaakt door een verhoogde migratie van urotheel cellen, in plaats van door een verhoogde proliferative activiteit. Urotheel dat groeit op laminine heeft maar een cellaag, die bestaat uit grote platte cellen. De geobserveerde versnelling in de uitgroei van het op laminine gekweekte primaire urotheel moet daarom worden toegeschreven aan het afplatten van epitheel cellen. Geconcludeerd kan worden, dat de groeisnelheid, differentiatie en de hoeveelheid cellagen van het primaire urotheel gemoduleerd kunnen worden middels groeifactoren en ECM componenten.

De waargenomen functionele effecten van groeifactoren en ECM componenten op primair urotheel zijn gevolgd door een studie naar hun invloed op de intra-epitheliale laterale infiltratie van T24 cellen in normaal urotheel. Deze experimenten zijn beschreven in hoofdstuk 4. Het toevoegen van EGF reduceert de mate van intra-epitheliale expansie van T24 cellen in primair urotheel: de hoeveelheid en de afstand van infiltrerende T24 cellen was hierdoor afgenomen. Als het primaire urotheel groeit in de aanwezigheid van aFGF of laminine neemt de hoeveelheid infiltrerende T24 cellen toe in vergelijking tot de niet behandelde kweken. Opvallend is, dat geen van de groeifactoren of ECM componenten de migratie en

proliferatie van de T24 cellen beïnvloedt. Als beschreven in hoofdstuk 3 moduleren de groeifactoren en ECM componenten de morfologie en de differentiatie van het primaire urotheel. Op grond hiervan moet het verschil in intra-epitheliale infiltratie door de T24 cellen in het primaire urotheel toegeschreven worden aan modulatie van het primaire urotheel, in plaats van aan een direct effect van groeifactoren en ECM eiwitten op de T24 cellen. Uit lichtmicroscopisch onderzoek kan opgemaakt worden dat er een omgekeerde relatie bestaat tussen de kerndichtheid van het primaire urotheel en de afstand en het aantal infiltrerende individuele T24 cellen.

In de experimenten beschreven in hoofdstuk 5 wordt het intra-epitheliale expansie patroon van E-cadherine positieve en negatieve blaaskanker cellen vergeleken. Enerzijds infiltreren E-cadherine negatieve cellijnen, zoals T24 het primaire urotheel als individuele cellen (pagetoide infiltratie patroon). Anderzijds infiltreren E-cadherine positieve tumorcellen als een aaneengesloten veld van cellen gepaard gaande aan een blokkade van de uitgroei van primaire urotheel. Dus, E-cadherine negatieve blaaskanker cellijnen infiltreren met een pagetoide infiltratie patroon, terwijl E-cadherine positieve cellijnen een uitbreidingspatroon hebben, dat dezelfde karakteristieken heeft als die van een carcinoma in situ.

In de experimenten, die zijn beschreven in de hoofdstukken 5 en 6 wordt het verspreiden en implanteren van tumor cellen op intact en getraumatiseerd urotheel bestudeerd. Meest opvallend is, dat alleen E-cadherine positieve tumor cellen kunnen hechten op intact muize-urotheel. E-cadherine negatieve tumorcellen zijn niet in staat om op intact urotheel te hechten. Ook niet als het urotheel door modulatie met groeifactoren en ECM componenten een veranderde morfologie heeft. De differentiatie van het primaire urotheel lijkt dus geen rol te spelen bij de hechting van de E-cadherine negatieve cellen. E-cadherine positieve tumorcellen, daarentegen, zijn wel in staat tot hechting op primair intact urotheel. Omdat de hechting van E-cadherine positieve tumorcellen aan intact primaire urotheel specifiek verhinderd kan worden met behulp van een antilichaam gericht tegen E-cadherine hebben wij geconcludeerd, dat de hechting van tumor cellen op intact urotheel E-cadherine afhankelijk is.

In hoofdstuk 6 is beschreven, dat E-cadherine negatieve T24 cellen kunnen hechten op kleine wondjes, die zijn geïntroduceerd in het urotheel. De gehechte T24 cellen infiltreren vervolgens het omringende urotheel. Deze infiltratie door T24 cellen in het regenererende

urotheel kan verminderd worden door toevoeging van EGF, zowel voor als nadat de verwondingen zijn aangebracht.

Conclusies:

1) Het hier beschreven in vitro cocultivatie model kan gebruikt worden om de mogelijke mechanismen, betrokken bij de vorming van recidief blaastumoren te bestuderen.
2) Groeifactoren en ECM componenten moduleren het pagetoide type infiltratie patroon van de E-cadherine negatieve T24 blaaskanker cellen, eerder door een indirecte interactie met het primaire urotheel dan door een direct effect op de tumor cellen.
3) E-cadherine expressie reguleert het patroon van laterale expansie van getransformeerde cellen.
4) Alleen E-cadherine positieve tumorcellen kunnen hechten op intact urotheel.
5) Groeifactoren beïnvloeden de uitbreiding van het met T24 tumorcellen bedekte oppervlakte in getraumatiseerde gebieden in het urotheel.

Verdere studies zijn vereist om de therapeutische mogelijkheden te onderzoeken, die gebaseerd zijn op de bevindingen van deze studie. Installatie van EGF voorafgaand aan de chirurgische verwijdering van een primaire blaastumor zou de recidief kans kunnen verminderen. De in vitro resultaten suggereren namelijk, dat toevoeging van EGF de intra-epitheliale infiltratie van E-cadherine negatieve tumor cellen reduceert en de regeneratie snelheid van het normale urotheel verhoogd. De rol van het calcium afhankelijke E-cadherine bij de hechting van tumorcellen aan intact urotheel zou eveneens op een klinische toepassing kunnen wijzen. Het recidiveren van E-cadherine positieve blaastumoren door implantatie van deze cellen op intact urotheel zou mogelijk verhinderd kunnen worden door eliminatie van Ca^{2+} met Ca^{2+} binders. Een alternatief om tumor implantatie in intact urotheel te verhinderen kan zijn met het gebruik van E-cadherine specifieke antagonisten.

CURRICULUM VITAE

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DANKWOORD

Nu het boekje in de definitieve vorm af, kan ik terug kijken op de afgelopen vier en een half jaar. Deze jaren zijn grotendeels gewijd aan mijn promotie onderzoek op de afdeling Pathologie. Hier heb ik met plezier gewerkt en er veel geleerd. Veel mensen hebben gezorgd, dat het werk toch altijd leuk was. Ze hebben mij zowel praktisch bijgestaan als wel gezorgd voor de nodige gezelligheid tijdens de koffie en borrels. De mensen, die op enigerlei wijze hebben bijgedragen aan dit proefschrift wil ik hierbij bedanken.

Er zijn enkele mensen, die ik graag met name wil noemen uit het "blaas groepje".

Lydia, zonder jouw inzet, enthousiasme en natuurlijk het "eeuwige" kweekwerk was dit boekje niet tot stand gekomen. Ik ben altijd erg blij geweest, dat we elkaar weer moed in konden spreken als onze celletjes weer niet blauw waren. Jij vond het geen probleem om na een kleine pauze, een appelflap of zo, toch gewoon weer verder te gaan.

Marcel, jou wil ik graag bedanken voor de enorme hoeveelheid blaasjes die jij in deze vier jaar voor mij op membranen hebt gezet. Ik ben de tel kwijt geraakt, maar het zijn er erg veel. Ook wil ik je bedanken dat je mij de immunohistochemie hebt bijgebracht en soms ook hebt uitgevoerd.

Mijn promotor, Theo, wil ik bedanken voor de begeleiding, je eeuwige enthousiasme en het altijd snel nakijken van manuscripten en brieven. Je hebt mij altijd enorm kunnen stimuleren niet op te geven en om resultaten positief te interpreteren. Ook wil ik Ellen bedanken voor de begeleiding en het altijd kritisch bekijken van resultaten. Hierbij wil ik ook Pim bedanken voor de uurtjes dat wij over de blaasjes in het algemeen gediscussieerd hebben.

Professor Bosman wil ik bedanken voor de belangstelling in dit onderzoek en voor de snelle afhandeling en het doorlezen van dit proefschrift. De leden van de kleine promotie-commissie wil ik bedanken voor het kritisch lezen van dit proefschrift.

Ook wil ik familie en vrienden bedanken voor hun steun en interesse. Als laatste, maar zeker niet als minst belangrijke, wil ik Peter bedanken. Je hebt meer voor mij gedaan dan je zou denken!