GROWTH FACTORS IN BLADDER EPITHELIUM

A study on the expression and functions of growth factors in mouse urothelium

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A study on the expression and functions of growth factors in mouse urothelium

GROEIFACTOREN IN BLAASEPITHEEL.

Een studie naar de expressie en functies van groeifactoren in urotheel van muizen

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C'est toi qui était entreé mon coeur à Paris;

Avec toi j'ai fini cette chapitre à Paris.

Aan Diana en Fabian.

Contents

BrdU	5-bromo-2′-deoxyuridine
BSA	bovine serum albumin
cpm	counts per minute
DAB	3,3′-diaminobenzidine
DNA	deoxyribonucleic acid
dpm	desintegrations per minute
DTT	dithiothreitol
EGF	epidermal growth factor
EGF-R	EGF receptor
FCS	foetal calf serum
FGF	fibroblast growth factor
FGFR	FGF receptor
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HGF	hepatocyte growth factor
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
kD	kilo dalton
LI	labelling index
LM	light microscopy
MAb	monoclonal antibody
PDGF	platelet-derived growth factor
RNA	ribonucleic acid
mRNA	messenger RNA
TCC	transitional cell carcinoma
TEM	transmission electron microscopy
TGF	transforming growth factor

The urogenital tract is one of the major excretory paths for small metabolites and ions. The excreted liquid, urine, is produced by the kidneys, flows through the ureters into the urinary bladder and is finally excreted through the urethra. The mammalian urinary bladder has the important capacity to retain urine for some time. Normally, the bladder is resistant to toxic effects of products in the urine. But after damage to the epithelium of the bladder, bladder epithelial cells may become vulnerable to xenotoxic agents and potential toxic metabolites in the urine. In general, this damage will be repaired by physiological processes. During neoplasia, e.g. in transitional cell carcinoma, aberrations in physiologically regulated processes occur. This thesis focuses on protein factors that may be involved in the physiology of transitional epithelium of the mouse bladder, and their specific functions. The following paragraphs highlight subsequently the structure and function of the bladder (§1), the physiology and causes of abnormal growth of the urothelium (§2), proteins that have been shown to be involved in the physiology of normal and tumour cells (§3), what is currently known about these proteins with respect to the bladder (§3.5), and finally the aim of the thesis (§4).

§ 1 *Structure and function of the bladder*

The urinary bladder is a vesicular organ. Figure 1 depicts the structure of the bladder. The lumen of the bladder is lined by transitional epithelium (also called urothelium). The outer part of the bladder wall is formed by a smooth muscle cell layer which is usually composed of an inner and an outer longitudinal layer and a circular middle layer. The submucosa or stromal compartment is located between the epithelium and the muscle layers. The stromal layer contains e.g. blood vessels, nerves, and cells of different types like fibroblasts, macrophages, and lymphocytes.

The urothelium consists of a basal lamina and several layers of epithelial cells. Murine urothelium has three to four cell layers, while human urothelium consists of up to 6 cell layers. The small, undifferentiated basal cells are in close contact with the basal lamina. The large, terminally differentiated umbrella cells are the superficial cells lining the bladder lumen. The cell layers between the basal and the superficial cell layer are called intermediate cell layers. The intermediate cells are larger than basal cells, and the more superficially positioned, the better differentiated these intermediate cells are. Table 1 summarizes some of the characteristics of these urothelial cells.

The main function of the bladder is to serve as a reservoir for urine before excretion. Upon a nervous stimulus the muscle cells contract the bladder for

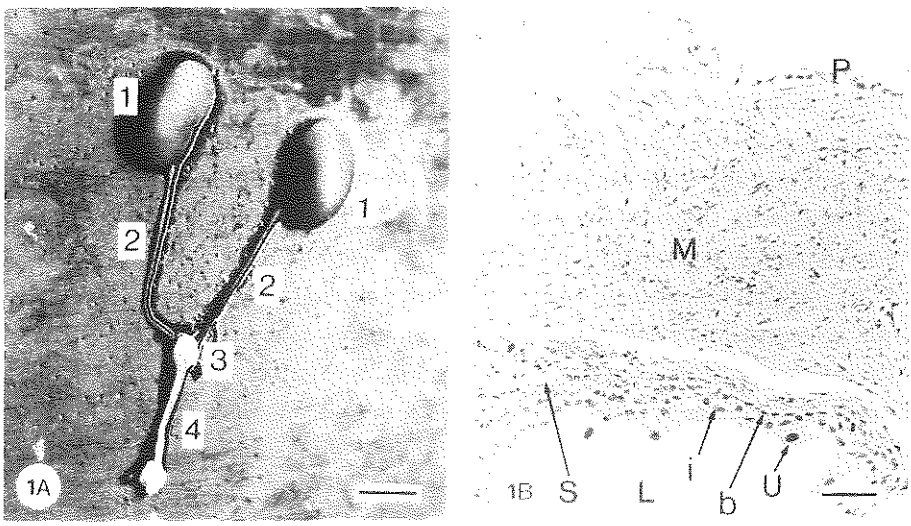

Figure 1. Structure of the urogenital tract (A) and the urinary bladder (B) of the mouse. A: The dissected ventral part of the urogenital tract of a female mouse. Numbers indicate the kidney (1), the ureter (2), the bladder (3), and the urethra (4). Bar = 14 mm. B: A haematoxylin-stained, paraffin-embedded cross section of a female mouse bladder. The different cell layers are indicated: P = peritoneum; M = muscle layer; S = submucosa; b, i = basal or inter-mediate urothelial cells, respectively; U = umbrella cells; L = bladder lumen. Magnification: 200 X; bar = 50 μm.

Table 1. Characteristics of urothelial cells of the respective cell layers.

cell layer	basal	intermediate	superficial
morphology	small, cuboid	larger, rectangular	very large, flat
ploidy	diploid	di- or tetraploid	up to more than octaploid
nuclei/cell	one	one or two	several
other features	hemi-desmosomes many free ribosomes few organelles alkaline phosphatase	desmosomes some autophagic bodies, Golgi vesicles alkaline phosphatase	desmosomes many autophagic bodies, vacuoles, specialized vesicles, tight junctions in lateral membrane ß-glucuronidase, acid phosphatase

Table 1. Main characteristics of urothelial cells in the respective cell layer of the murine urothelium.

micturition. The urothelium is a dynamic structure because it should be resistent to mechanical stress due to the continously repeated process of filling and emptying of the bladder. Both the junctional complexes (zonulae occludentes) interconnecting umbrella cells, and the desmosomes between urothelial cells form the tight cohesion between urothelial cells. The plasticity of the urothelium is provided by specialized areas in the luminal membranes of umbrella cells [101]. In short, umbrella cells have numerous small vesicles positioned perpendicular to the luminal membrane. Upon filling of the lumen, these vesicles fuse with the luminal membrane thereby enlarging the luminal membrane area. Upon micturition, these vesicles are formed again, serving as a reservoir for enlarging of the luminal membrane. Both micturition and the control over this mechanism serve several functions: 1) reduction of the risk of ascending bacterial infections from the urethra by keeping the external body surface clean and dry, 2) the use of urine in combination with pheromones by some animals e.g. for territorial demarcation, 3) to avoid fouling of the nests, holes, houses, etc., 4) to avoid the attraction of predators via pheromones in the urine.

The urothelium is in direct contact with the urine. The urothelium is a barrier to the hypertonic urine. The urothelium is impermeable to water and urinary products like ions, ureum, small proteins, and metabolized and/or detoxified products [101]. In order to achieve this impermeability, the urothelial cell membrane contains cerebroside [128]. This is a specific feature of urothelial cells since cerebroside has not been found in membranes of other epithelial cells. Cerebroside is shown to decrease the permeability of the plasma membrane for water [100], and to increase its electrical resistance [54]. Despite the impermeability, urinary products can be ingested by the urothelial cells via the formation of intracellular vesicles after micturition, or can be swallowed by phagocytosis [101].

§ 2 *Physiological and pathological processes*

§ 2.1 *Physiology of the urothelium*

Since the main function of the bladder urothelium is to provide an impermeable barrier to urine, any damage to the urothelium will affect its impermeability. Upon injury, the urine with a variety of molecules penetrate into the bladder wall. Such a damage may be caused by catheterization, intravesical operations, or overstretching of the bladder as a result of bladder outlet obstruction. Loss of urothelium followed by a complete self-renewal also occurs physiologically in neonate mice [16,76]. Epithelial wound healing occurs by both cell divisioning and migration of epithelial cells. Cell division in the murine urothelium occurs mainly in the basal cell compartment of the urothelium and, rarely, in the intermediate cell layers. Under normal conditions cell

divisions are hardly seen and cell cycle times are high, i.e. over 60 hours [123], resulting in a low turn-over of urothelial cells. It takes more than 200 days for a basal cell before it is differentiated into an intermediate cell, and finally shedded as umbrella cell [123]. Though, if damaged urothelium regenerates, many epithelial cells in basal, intermediate, and even superficial layers start to proliferate and the cell cycle time decreases to less than 28 hours. Both the enhanced proliferation and the concomiting migration of urothelial cells result in a rapid re-epithelialization [101]. This points to the enormous proliferation capacity of the urothelial cells necessary for wound healing and restoration of function. In addition, the damaged urothelium can also be re-epithelialized by transitional epithelial cells from the ureters and urethra, by proliferation and migration [101].

§ *2.2 Pathology of the urothelium*

Damage to the urothelium or aberrant growth of urothelium can be caused by disease processes in the bladder. Damage to the urothelium may lead to cell death and subsequent repair, but may ultimately also result in neoplasia. This paragraph describes four different ways how abnormal urothelial growth or urothelial cell death can be achieved.

Cell death Bacteria may ascend through the urethra in the reverse direction into the bladder lumen and cause a bacterial cystitis. Aronson et al [12] showed that intravesical application of E. coli caused shedding of normal murine urothelium in vivo one hour after infection. This effect might be explained by a direct cytotoxic action by bacteria on urothelial cells. In support of this direct cytotoxic action, Lee et al [143] noted that intravesical E. coli infection led to necrosis of tumour cells in the bladder.

In addition, the anti-tumour activity of bacteria is also used in the treatment of transitional cell carcinomas (TCC) of the bladder. TCCs are malignant tumours of the urothelium. In order to reduce the recurrence rate of a TCC, patients may be treated with Bacille Calmette-Guerin (BCG) after a transurethral resection [120,202]. BCG is an attenuated strain of the bacteria Mycobacterium bovis and stimulates both the immune system and the anti-tumour activity [reviewed in 120,203].

As outlined below, cytotoxic agents may also cause erosion of the urothelium without concomitant neoplasia.

Hyperplasia Studies with murine urothelial cells showed that intravesical instillation of Escherichia coli bacteria or E. coli-derived lipopolysaccharides in vivo can induce hyperplasia of the urothelium within one week [60,234]. Since both bacteria and LPS stimulate immunological defense mechanisms [120,202], it has been suggested that the urothelial hyperplasia is induced by lymphocyte- and/or macrophage-derived

cytokines [234].

Metaplasia Urothelial aberrations with loss of function can be caused by small crystals and calculi in the urine [57]. Continous exposition to these crystals or calculi causes squamous metaplasia: the transitional epithelium is replaced by a squamous cell epithelium. This results in a loss of the barrier function. Metaplasia with loss of urothelial function also occurs after infection with a worm, Schistosoma haematobium. Schistosomiasis of the bladder has also been shown to be strongly associated with the occurrence of TCC [84,119].

Neoplasia Neoplasia of the urothelium can be caused by compounds of e.g. food, drugs, or tobacco smoke. Previous studies indicated that the chemotherapeutical drugs cyclophosphamide and adriamycin are cytotoxic to murine urothelium [57,145]. Other agents are thought to be associated with TCC, like abuse of the analgetic drug phenacetin [161], or aromatic amines like 4-aminobiphenyl, 2-naphtylamine, and benzidine which are present e.g. in tobacco smoke or in the occupational environment [119,163]. In general, such agents are metabolized in the liver and conjugated by glucuronidation, sulfation, or acetylation. Once these metabolites or non-metabolized compounds have been excreted in the urine, they can be internalized by urothelial cells. It is suggested that some of these metabolites enter the superficial urothelial cells by being endocytosed in membrane vesicles after micturition [101]. These vesicles may fuse with lysosomes, which contain ß-glucuronidases that uncouple the glucuronic acid group. Parallel to this proposed mechanism, metabolization may also occur by urinary bacteria. In addition to glucuronidases, urothelial cells also contain other metabolizing enzymes like cytochrome P450 isoenzymes [199,239], prostaglandin H synthase [reviewed in 122], sulfatases [36], and deacetylases [172], each of which may potentially activate the toxic urinary compounds. The activated metabolites may either be genotoxic (e.g. 4-aminobiphenyl [199], benzidine [122,163], phenacetin [161]) or cytotoxic resulting in urothelial erosion and/or neoplasia (e.g. cyclophosphamide and its metabolites acrolein and phosphoramide mustard [57,112,145]).

§ 3 *Growth factors*

§ 3.1 *Growth factors: Introduction*

During the past two decades many studies have been performed investigating the molecular basis of wound healing, tumour growth, and embryogenesis. In some of these studies polypeptide growth factors were involved. Growth factors are highly conserved between species. For example, the amino acid sequence of insulin-like growth factor I was conserved for 77% for species ranging from salmon to human [210], suggesting their important function.

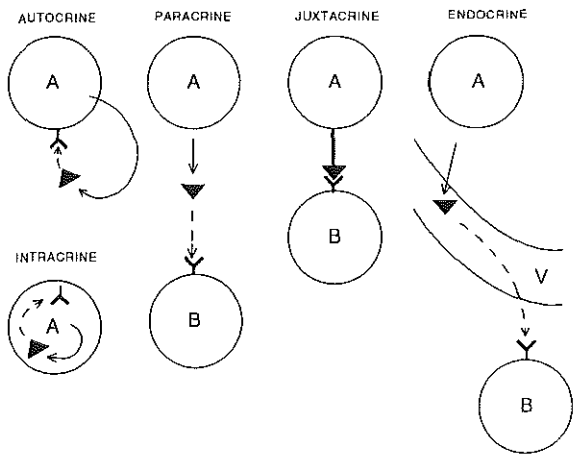

Figure 2. Mechanisms of growth factor action on cells. Secreted or membrane-bound growth factors are represented by free and fixed triangles, respectively. The growth factors bind to cellular receptors (represented by Y-shaped symbols). V = blood vessel

Growth factors exert their effects on cells through specific, cell membrane bound receptors which transduce their intracellular signals via second messengers. While the classical hormones have distant, systemic effects, growth factors can act locally at or near the site of secretion. The following mechanisms for growth factor actions on cells can be distinguished: 1) autocrine including intracrine, 2) paracrine, 3) juxtacrine, and 4) endocrine (see Figure 2).

As shown in Table 2, a number of families of growth factors has been identified. Within each family the members share similar features like homology in cysteine sequence and their tertiary structure, binding to heparin (heparin-binding growth factors or fibroblast growth factors (FGF)), chemico-physical similarity, and functional effects. Although much attention has been paid to e.g. skin, trachea, and intestinal epithelial cells, little is known about the function of growth factors in urothelium. However, several findings strongly suggest a specific role for growth factors in the maintenance and repair of normal urothelium, and neoplastic urothelial disease: normal urine contains epidermal growth factor (EGF) and transforming growth factor ß (TGFß); urine of patients with TCC contains TGFα and FGF-like molecules; FGF-1 is present in TCCs but not in normal urothelium; and the expression of EGF receptors seems to correlate with an increasing aggressiveness of TCC. This chapter addresses possible functions of

growth factors and their receptors in (epithelial) cells in vitro and in vivo.

§ *3.2 Growth factors: Structure and function*

Table 2 lists most growth factor families that have been shown to be important for epithelial cells. The cellular and tissue specific localization and some of the functional aspects of the individual members are also given.

EGF The epidermal growth factor family includes EGF, TGFα, heparin binding EGF (HB-EGF), and amphiregulin. These proteins are structurally homologous in having 6 cystein residues at similar positions with disulphide bonds. The overall amino acid homology between EGF and TGFα is approximately 42% [63]. EGF-like proteins are heat and acid resistant, but can be inactivated by agents that reduce the disulphide bonds. Less is known about the 22 kD heparin binding EGF and the 9.8 kD amphiregulin. Heparin binding EGF and amphiregulin are unique in this family for their ability to bind to heparin [26,102,201], in contrast to EGF and TGFα. Since EGF and TGFα have been studied most, this thesis will focus on EGF and TGFα. The 6 kD EGF protein is most abundant in the mouse maxillary and submandibulary glands, and in human and murine urine and milk [42,104]. TGFα is present in other tissues than EGF, except for epidermis (see Table 2). The functions of EGF and TGFα are quite similar, as shown for the eye-lid reflex [64] and several cellular responses in vitro (see Table 2). However, in general, TGFα is a more potent growth factor than EGF as has been demonstrated for e.g. proliferation and migration of keratinocytes [18]. Many studies on EGF/TGFα function have been performed in skin wound healing experiments in vitro and in vivo. Both EGF and TGFα can accelerate the wound healing of skin wounds by topical application or via systemic delivery through an intraperitoneal minipump [38,184,216,217]. According to results from in vitro experiments with skin explants and isolated keratinocytes, EGF/TGFα are thought to accelerate the migration of keratinocytes at the wound edge down the edge of the fibrin clot resulting in the closure of the wound [26,155]. In addition, the multiple cell layering of the epidermis is probably a result of an EGF-mediated multiplication of the keratinocytes since in vitro studies indicated that EGF/TGFα stimulates the proliferation of keratinocytes [18,93,249]. In support of the proliferation-inducing capacity of EGF, Messing et al [165] showed that intravesically instillated EGF in the lumen of rat bladders increased the [^{3}H]-thymidine uptake in urothelial cells. Another study, using transgenic mice overexpressing TGFα, suggested that TGFα is capable of inducing proliferation of mammary epithelial cells [117]. The latter study also showed that TGFα is a protein with transforming capacity, inducing hepatocarcinomas. Several studies indeed

Table 2. Growth factors, localization, and function.

TYPES	LOCALIZATION	FUNCTION					REFERENCES
		Prolif	*Differ*	*Migr*	*Metab*	*RNA,protein*	
EGF	kidney,salivary glands,epidermis,	ep,fb,en,hep,	ep	ep,fb	ep,hep	ep,hep,fb,en,	18,28,42,45,61,73,
	renal + bladder carcinoma	cho,smu			fb	os,cho	90,142,185,223
TGFα	epidermis,brain*,activated macroph.,placenta,	ep,fb,en,smu		ep		ep	18,42,55,64,248
	ovary,embryo,carcinomas						
HB-EGF	activated macroph.,lung*,brain*,heart*, muscle	ep,fb,smu,ly		smu			102,240
amphiregulin	epidermis*,ovary*,colon*,kidney*,carcinomas	ep		ep			193,195
TGFß$_1$, TGFß$_2$, TGFß$_3$	platelets,activated macroph.,lymphocyt,epidermis,	ep,fb,en,ly,	ep,ly,	ep,fb,	ep,en	ep,fb,en,mu,cho	21,23,29,31,61,109,
	cartillage,kidney,placenta,colon,embryo,carcinoma	os,cho,smu	mu	mon			138,140,158,206
MIF	male foetal testes						44
activins, inhibins	gonads						131
FGF-1 (aFGF)	brain,retina,macroph.,bone,kidney,uterus,	ep,fb,en,smu,	en,fb,	ep,en		ep,en,cho,mu	28,31,132,204
	smooth muscle cells,carcinoma	os,cho,hep,neu	neu,cho				
FGF-2 (bFGF)	ubiquitous,embryo,carcinomas	mel; see aFGF	see aFGF		en,fb	ep,fb,en,cho,mu	28,31,58,61,94,103,132, 146,183,192,201
FGF-3 (INT-2)	embryo,carcinomas	ep,fb					66,114,176,192
FGF-4 (K-fgf/HST-1)	colon mucosa,embryo,Kaposi sarcoma,carcinomas	ep,fb,en,mel	neu				62,66,80,192,252
FGF-5	neuronal tissue,embryo,tumours	ep,fb,en					22,66,92,94,254
FGF-6 (HST-2)	skeletal muscle*,embryo*,tumours*						88,154
FGF-7 (KGF)	fibroblasts,kidney,colon,ileum,dermis	ep					75,212
FGF-8	mammary carcinoma cell line SC-3	SC-3					227
FGF-9	glioma cell line,brain*,kidney*	fb,gl					167

Table 2. continued

TYPES	LOCALIZATION	FUNCTION					REFERENCES
		Prolif	*Differ*	*Migr*	*Metab*	*RNA,protein*	
PDGF-AA	smooth muscle cells,osteosarcomas,melanoma	ep,fb,smu,neu,	SK,LE	ma,fb,	en,fb	fb	8,23,28,29,37,
PDGF-BB	platelets } PDGF-B RNA in epidermis,kidney,	en		smu			52,72,77,85,
PDGF-AB	platelets } mesothelioma,carcinoma,sarcoma						97,155,208,209
IGF-I	liver,fibroblasts,cartillage,granulosa cells,	ep,fb,cho,neu	ep,os,	ep	fb,en,ad,	fb,cho,neu,os,	7,28,52,61,78,
	brain explants,embryo,tumours		mu		mu,neu	hep	79,103,126
IGF-II	muscle,brain,liver,nerves,skin,adrenal gland, T.-I.	ep,fb,cho,neu	mu,ov,	mu	mu,hep	neu,os	28,61,78,
	cells,kidney,cerebrospinal fluid,embryo,tumours		os		neu		79,186
relaxin	corpus luteum						39
insulin	pancreas,serum	ep,hep,fb	ep,mu		hep,fb,	hep,fb,mu	28,45,47,79,111,
					ad,mu		126,168,186
HGF	platelets,liver,lung,kidney,brain,exocrine pancreas,	ep,hep	ep	ep,hep			13,99,170,226
	thyroid,salivary glands,Brunner's glands						

Table 2. Partial outline of the growth factor families and their respective members, the in vivo or in vitro localization, and their ability to modulate the cellular proliferation (Prolif), differentiation or maturation (Differ), migration (Migr), metabolism (Metab), and synthesis of macromolecules like RNA or proteins. The functions of the subtypes of TGFß and PDGF, respectively, as well as the localization of TGFß subtypes, are taken together. Legend: ad = adipocyte, cho = chondrocyte, en = endothelial cell, ep = epithelial cell, fb = fibroblast, gl = glia cell, hep = hepatocyte, ly = lymphocyte, ma and macroph. = macrophage, mel = melanocyte, mon = monocyte, mu = muscle cell, neu = neurectodermal cell, os = osteoblast, ov = ovarian granulosa and thecal interstitial cell, smu = smooth muscle cell; LE = lens epithelium, SK = skin, T.-I. = thecal-interstitial; * = RNA.

demonstrated the transforming capacity of TGFα: Ju et al [125] and Di Marco et al [153] showed that 3T3 fibroblasts expressing EGF receptors acquired a transformed malignant phenotype upon transfection with TGFα cDNA, while treatment of normal rat bladder explants and rat urothelial cell lines with EGF/TGFα in vitro also induced morphological characteristics of neoplastic cells [134,254].

A biological difference between expression of EGF and TGFα can be noted in embryos. While EGF is present in neonate and adult tissues, it has not been detected in foetal tissues [198], in contrast to TGFα [248]. This suggests a role for TGFα but not for EGF in embryonal development.

TGFß The transforming growth factor ß superfamily has many members [reviewed in 131,159], including the TGFß subfamily, the inhibin/activin subfamily, and the bone morphogenetic proteins (BMPs) (Table 2). Since TGFßs are known to be generally expressed in epithelial cells, we will focus here on the TGFß subfamily. Five members of the TGFß subfamily have been identified of which TGFß$_1$, TGFß$_2$, and TGFß$_3$ have been found in mammalian species. Both TGFß$_2$ and TGFß$_5$ have been found in Xenopus laevis [207], but a mammalian homologue of TGFß$_5$ has not been found yet. TGFß$_4$ was originally isolated from chicken [115]. TGFßs are synthesized and secreted as inactive precursor molecules, which are cleaved to render mature carboxy-terminal molecules of 110-140 amino acids [89,131,229]. The N-terminal pro-domain of TGFß can also remain associated with the carboxy-terminal molecule resulting in a latent TGFß-complex. This complex is activated enzymatically, or by heat or acid treatment, which dissociates the mature TGFß from the pro-domain. Treatment with reducing agents inactivates TGFß [89,131]. The activated TGFß is a homodimeric protein of 25 kD.

TGFßs are known for their ability to inhibit the EGF-induced proliferation of epithelial cells in vitro. In contrast, TGFß acts as a bimodal regulator of the proliferation of smooth muscle cells, fibroblasts, and chondrocytes in vitro: low concentrations of TGFß induce the proliferation, while higher concentrations inhibit the proliferation [23]. The mechanism for this bimodal regulation of proliferation in connective tissue cells has been assigned to its interaction with a PDGF autocrine loop. Battegay et al [23] demonstrated that fibroblasts secreted PDGF-AA and expressed the PDGF type α receptor in vitro. At lower concentrations TGFß induces secretion of PDGF-AA which is mitogenic for connective tissue cells. At higher concentrations TGFß down regulates the PDGF type α receptor expression resulting in a decrease of the response to PDGF-AA [23]. Studies on the TGFß-inhibitory mechanism in epithelial cells are now in progress. Both the transcription factor c-*myc*, which is involved in proliferation, and the proliferation suppressing retinoblastoma gene product, pRB, may be involved [175].

Recent studies demonstrated that TGFß inhibits both the expression of the transcription factor c-*myc* and the proliferation of keratinocytes equally well as antisense c-*myc* oligonucleotides [194]. From other studies it is now proposed that TGFß inhibits the activation of pRB and the transcription of c-*myc*, either directly or via a cascade of other proteins [175,194].

Besides proliferation modulation, TGFß has also been shown to be involved in cartilage formation by inducing differentiation of chondrocytes and secretion of extracellular matrix proteins [31,89]. Studies with epithelial cells demonstrated that TGFß can also induce terminal differentiation of rat tracheal cells and murine and human keratinocytes in vitro [86,213,229,249].

Other members of the TGFß superfamily are 1) the TGFß-related Müllerian Inhibiting Factor which is expressed in foetal male testes and induces regression of the Müllerian duct [44]; 2) inhibins and activins; 3) the decapentaplegic protein DPP-C in Drosophila; and 4) the Vg-1 protein in Xenopus laevis embryos. The activin subfamily consists of homo- or heterodimers of the A (or inhibin ß A) and the B (or inhibin ß B) subunit [131,159]. Activins and inhibins are synthesized in the gonads. They are implicated in the stimulation or inhibition, respectively, of the pituitary Follicle Stimulating Hormone (FSH) secretion, and the induction of mesodermal development.

FGF The fibroblast growth factor family is expanding: during the last five years several new members have been found. Five FGF proteins were shown to bind to heparin or heparan sulphate chains: FGF-1, FGF-2, FGF-4, FGF-5, and FGF-7 [192,212,255], while FGF-3 does not bind to heparin [192]. In contrast to FGF-1, FGF-2 does not require exogenous heparin for its proliferative action on cells in vitro, although Yayon et al [252] showed that heparin or heparan sulphate chains enhance the interaction of FGF-2 and its receptor. The overall homology of FGF proteins compared with FGF-2, excluding the N- and C-terminal parts, ranges from 39% (FGF-7) to 55% (FGF-1) [reviewed in 25]. Structural differences have also been noted: in contrast to other FGFs, both FGF-1 and FGF-2 lack a signal sequence for secretion [2,25, 226,132], implicating a predominantly intracellular function for FGF-1 and FGF-2. However, FGF-1 and FGF-2 have also been detected in the extracellular matrix. Several mechanisms have been put forward to explain their extracellular localization: 1) disruption of cells [162], 2) co-transport with heparin sulphate chains [132], 3) exocytosis or evagination of specialized cellular compartments, or 4) active transport across the plasma membrane [127].

As listed in Table 2, FGFs regulate a variety of processes in epithelial cells. Several FGFs, like FGF-1 and FGF-2, localize in both embryonal and adult tissue [130,

Table 2]. In contrast, FGF-3 RNA is expressed only in embryonal tissues suggesting a specific role for FGF-3 in embryogenesis [250]. FGF-3 to -5 were originally detected in tumours, using a fibroblast transformation assay. FGF-3, FGF-4, FGF-5, and FGF-6 are capable of transforming 3T3 cells morphologically in vitro [22,62,66,154]. FGF-3 to -5 were able to induce the proliferation of epithelial cells in vitro [66]. Moreover, transgenic mice expressing FGF-3 showed hyperplasia of prostate (male mice) or mammary gland (female mice) epithelium [176]. These data support a potential role for FGF-3 in epithelial proliferation and aberrations in epithelial growth. Some studies indicated tissue specificity in expression patterns of FGFs. While FGF-1 and FGF-2 are synthesized by both epithelial and non-epithelial cells, Werner et al [247] demonstrated that FGF-7 is only expressed by non-epithelial cells. The FGF-7 receptor was present only on epithelial cells suggesting a paracrine action of FGF-7 on epithelial cells. Studies of Finch et al [75] and Rubin et al [212] showed that FGF-7 indeed acts through paracrine mechanisms on epithelial cells.

FGFs are also considered as angiogenic factors inducing angiogenesis and neovascularization in vivo [reviewed in 132,201]. Several in vivo experiments indicated that FGF-1 and FGF-2 induce the formation of capillaries in wounds and tumours. Hori et al [106] showed that neovascularization of tumours in vivo could be inhibited by a neutralizing antibody against FGF-2. Moreover, this inhibition resulted in suppression of tumour growth [106]. Other studies provided indirect evidence that FGFs are angiogenic proteins. In vivo studies pointed out that de novo vascularization was stimulated by exogenous addition of FGF-1 or FGF-2, or by endogenous overexpression of FGF-1 in endothelial cells transformed with the FGF-1 gene [127,133,178]. A role of FGF in the induction of angiogenesis by direct action on endothelial cells is further substantiated by in vitro studies showing that FGF-1 and FGF-2 can stimulate endothelial cells to proliferate and to migrate [25,29,132,201]. However, FGFs are not unique in being angiogenic since other growth factors, including TGFß, also have angiogenic properties [133,151,205].

Recently, new members of the FGF family have been identified like Xenopus embryonic FGF, XeFGF, which has been found during embryonic development of Xenopus laevis. XeFGF has homology to FGF-4 and FGF-6 and exerts a mesoderm-inducing activity [110]. Other members are androgen-induced FGF-8 and glia-activating FGF-9 with 30-40 % homology to other FGFs [167,227]. The biological functions of these new members of the FGF-family are not yet fully elucidated.

PDGF Platelet-derived growth factor was initially purified from porcine and human blood platelets. The growth factor is a homo- or heterodimer of PDGF-A or PDGF-B chains

linked by disulphide bridges [209]. The PDGFs are known for their mitogenic action on fibroblasts and glial cells [52,96,209], while they are chemotactic for fibroblasts, smooth muscle cells, monocytes, and neutrophils [87,218]. The molecular weight varies from 24 kD (cell-associated form of PDGF-BB) to 30 kD (PDGF-AA, PDGF-AB, and the secreted form of PDGF-BB) [191]. With respect to epithelial tissues, little is known about the function of PDGF.

Recent studies gave evidence that only under certain conditions PDGFs are expressed in epithelial cells: e.g. in epidermal cells after skin injury [8,10], in renal visceral epithelial cells during renal glomerular nephritis [77], in lung and gastric carcinomas [9,27,51,215], and in prostate, breast, gastric, colon, and thyroid carcinoma cell lines [11,97,215,220]. In addition, PDGF B-chain which is encoded by c-*sis* [121], is functionally identical to the transforming protein of the Simian sarcoma virus $p28^{sis}$ [246]. These studies suggested a role for PDGF in tumorigenesis.

Paracrine mechanisms of PDGF have been suggested by e.g. Barreca et al [19]: epithelium-derived PDGF may act on surrounding mesenchymal cells which express PDGF receptors. Upon PDGF-stimulation, these mesenchymal cells may synthesize and secrete IGFs which in turn act on epithelial cells. Other studies suggest that PDGFs act also in an autocrine way on regenerating keratinocytes in vivo [8] or on carcinoma cells [9,51,97]. A number of studies point to a function for PDGF in epithelium: PDGF was reported to stimulate the maturation of lens epithelium in vitro [37], and to enhance the migration of human retinal pigment epithelial cells in vitro [41] and of keratinocytes in vitro [71]. PDGF-BB also stimulates the proliferation of normal mammary cells and mammary carcinoma cells in vitro [70,228]. In vivo studies on skin wound healing showed that exogenous PDGF increased the re-epithelialization, suggesting that PDGF acts on migration and/or proliferation of keratinocytes [151,177]. PDGF can induce the synthesis and secretion of IGF-I in fibroblasts in vitro [52], which is mitogenic for keratinocytes in vitro (see IGF section). Hence, it cannot be excluded that the enhanced re-epithelialization by exogenous PDGF during wound healing in vivo was caused by an enhanced production of stromal-derived growth factors, like IGF-I, which act via paracrine action on keratinocytes.

IGF Insulin-like growth factors I and II show structural homology to insulin. The amino acid sequences are about 50% homologous with proinsulin [82,187,210]. In general, both proteins have similar insulin-like activities in cells (see Table 2), though IGF-I is more potent than IGF-II in vitro [187]. Furthermore, the expression patterns differ, especially in rat foetal tissues where IGF-II is expressed more abundantly than IGF-I [82]. Similarly, in the adult rat oval IGF-I mRNA is only found in granulosa cells

and IGF-II mRNA only in thecal-interstitial cells [105,210]. In contrast to IGF-II, expression of IGF-I in vivo is regulated by growth hormone [reviewed in 79,105].

In vitro studies showed that some cells need a competence factor like EGF, FGF, or PDGF for transition from G0 to G1 phase of the cell cycle, and for progression through the G1 phase [1,46]. In addition, for further progression through the late G1 phase, cells only require IGF or insulin in vitro [46,150]. Supporting in vivo evidence for these in vitro experiments was given by Lynch et al [151] who demonstrated that only the combination of PDGF-BB and IGF-I, but not the single factors, led to a higher regeneration rate of skin wounds in vivo. This might have been due to a direct effect of the exogenous IGF on keratinocytes [151]. Circumstantial evidence resulted from in vitro experiments, showing that both types of IGF stimulated the proliferation and migration of human keratinocytes [7,19,61,135], and that IGF-I was also able to induce differentiation of transformed human keratinocytes [126].

Other in vitro studies demonstrated that growing cultures of fibroblasts from normal tissue produced IGF-I [19,59] and fibroblasts from breast tumours produced IGF-II [59]. The latter authors suggested a growth promoting role for IGFs on breast epithelial cells, and especially a role for IGF-II in malignant lesions. Barecca et al [19] presented data indicating that the fibroblast-derived IGF could induce the proliferation of human keratinocytes in a paracrine way. In addition to the ability of IGF to induce differentiation in vitro, Matejka and Jennische [160] showed that after hypoxia IGF-I mRNA and protein were detectable in regenerating rat kidney tubular cells in vivo, concomittant with cell differentiation rather than proliferation. Other studies pointed out that IGF mRNA expression is not changed during wound healing [3,10].

IGF effects on cells not only depend on the presence of specific receptors on the cell membrane, but are also regulated by small binding proteins called insulin-like growth factor binding proteins (IGFBP). At the moment, 6 different IGFBPs have been found [reviewed in 53,67]. One of the functional aspects of some IGFBPs is to inhibit the binding of IGF-I to its receptor by binding the IGF-I. Albiston et al [3] showed that after injury of the rat ileum in vivo, the IGF-I and the type I IGF receptor expression did not change but IGFBP-3 mRNA levels decreased. This suggested that IGF might stimulate wound healing not by increasing IGF expression but as the consequence of a decrease in IGFBP and therefore an increased availability for binding to its receptor.

HGF Hepatocyte growth factor, or scatter factor, has been found during the regeneration of liver after partial hepatectomy [179] or liver injury [13]. It is an 85 kD heterodimer protein consisting of a smaller 34 kD and a larger 69 kD subunit which are linked by disulphide bridges. The protein binds to heparin [13], but shows no homology

with any FGF [180]. Previous studies showed that HGF is a motility and proliferation factor for epithelial cells [226]. Recent studies indicated that HGF is a paracrine acting factor produced by mesodermal cells in a variety of organs or tissues and acting on epithelial cells (see Table 2).

In conclusion, several growth factors show overlapping activities in modulating epithelial proliferation, differentiation, and migration. TGFß is different from EGF-like, FGF-like, PDGF-like, or IGF-like proteins in that TGFßs inhibit the proliferation of most examined epithelial cells.

In general, TGFα may be important for embryonal development and growth of carcinomas since TGFα is predominantly expressed in embryos and carcinomas, while EGF has a physiological function in neonates and adults (Table 2). In adult epidermis, both EGF and TGFα are expressed. Probably, EGF and TGFα stimulate epidermal wound healing by enhancing epithelial proliferation and migration ([38], Table 2). In vivo studies on RNA and protein expression, as well as functional studies based on exogenous application of growth factors in vivo suggested that TGFßs and FGFs are implicated in several physiological processess e.g. in embryogenesis, and in epithelial wound healing [10,130,151,177,247]. Data from in vitro studies suggest that 1) epithelial proliferation is induced by FGFs and EGFs, 2) epithelial differentiation and extracellular matrix formation can be induced by TGFßs, 3) migration is stimulated by EGFs, FGFs, and TGFßs, and 4) angiogenesis is induced by FGFs. Since the expression of FGFs is enhanced in several carcinomas, including human TCCs [50], FGFs may also have a function in the growth of carcinomas by an auto- or paracrine stimulation of the proliferation of tumour cells, and induction of tumour vascularization. IGFs are important as cell cycle progression factors and function therefore both in physiological and in tumour growth. IGFBPs can regulate the effects of IGFs by binding of IGFs preventing thereby the interaction of IGFs with IGF receptors. Finally, PDGF receptors have been found to be expressed in some epithelial cell types under certain conditions like wound healing or in carcinomas. Only a few in vitro studies demonstrated that PDGFs may stimulate proliferation, migration, or differentiation of some epithelial cell types in vitro. Therefore, little is known about general functions of PDGFs in epithelial cells.

§ *3.3 Growth factors: Growth factor receptors*

Growth factors exert their effects on cells via specific transmembrane receptors. The structure of some growth factor receptors is depicted in Figure 3. Most receptors have an extracellular binding domain for their ligand, a

transmembrane domain, and an intracellular protein kinase domain. Of these receptors, only the TGFß receptors do not have a tyrosine kinase but a serine/threonine kinase domain. Upon binding of the ligand, the receptors for EGF, FGF, and PDGF di- or oligomerize and are internalized [235]. Table 3 summarizes some of the characteristics of these receptors.

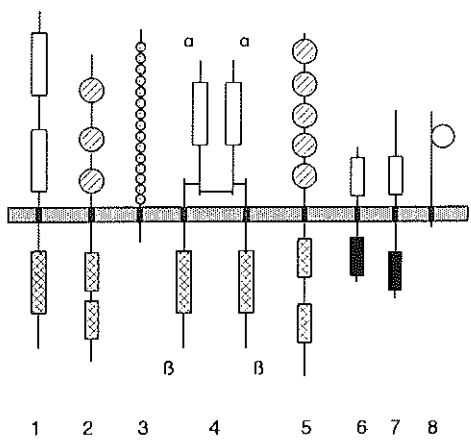

Figure 3. Structure of different families of transmembrane growth factor receptors. Numbers refer to the EGF receptors (1), FGF receptors (2), type II IGF receptor (3), the insulin and type I IGF receptors (4), PDGF receptors (5), and the TGFß type I (6), type II (7), and type III (8) receptor. Intracellular domains are represented by cross-hatched (tyrosine kinase domains) or black boxes (serine/threonine kinase domains). Extracellular domains are represented by open boxes (cysteine-rich domains), hatched circles (immunoglobulin-like domains), small stippled circles (cysteine-rich repeat sequences), or an open circle (glycosaminoglycan chains).

The EGF receptor family consists of c-erbB-1 to c-erbB-4 proteins. The c-erbB-1 proteins are the classical EGF receptors binding both EGF and TGFα equally well. Several studies indicated that two affinity classes of EGF receptors exist: receptors with a high or a low affinity for EGF/TGFα of which high affinity class receptors are thought to be necessary for signal transduction [24]. The expression of EGF receptors can be regulated by factors like EGF/TGFα, bFGF, or TGFß [14, 136,152,157].

The c-erbB-2 protein, also called neu or HER-2, is a protein with proto-oncogenic properties. Amplification or point mutation of the c-erbB-2 gene has been found in a variety of tumours, including bladder carcinomas. Both the overexpression of c-erbB-2 and the amplification or mutation of the c-erbB-2 gene correlated with a less favourable

Table 3. Characteristics of growth factor receptors.

RECEPTOR	LOCALIZATION	SPECIFICITY	REMARK	REFERENCES
EGF-R				
c-erbB-1	ubiquitous	EGF,TGFα,AR,HB-EGF	TM	10,42,165,
c-erbB-2	carcinoma,embryo	?	TM	43,173
c-erbB-3	GIT,UT,skin,lung,carcinoma, embryo	heregulins	TM	43,197,200, 214
c-erbB-4	heart*,muscle*,brain*,kidney*, lung*,carcinoma*		TM	43,196
TGFß-R				
type I			TM	26,68,201
type II	ubiquitous,carcinoma,embryo	TGFßs	TM	26,68,201
type III			NKD	26,201
FGF-R				
FGFR1	brain,heart,muscle,kidney, lung,carcinoma,embryo mesenchyme		TM	107,118,247
FGFR2	brain,skin,kidney,lung, carcinoma,embryo epithelium	see text	TM	118,247
FGFR3	brain,skin,kidney,lung,embryo endoderm		TM	118
FGFR4	liver,adrenals,kidney,lung		TM	118,224
PDGF-R				
type $\alpha\alpha$	mesenchymal cells,olfactory	PDGF-AA,PDGF-AB	TM	91,144,242
type αß	epithelium*	PDGF-AA,PDGF-AB	TM	91
type ßß	epidermis*,tumours	PDGF-BB	TM	8,9,91,242
IGF-R				
type I		IGF-I,IGF-II,(insulin)	TM	82,187,219
type II	ubiquitous,tumours,embryo	IGF-I,IGF-II	NKD	82,187,219
insulin		IGF-I,IGF-II,insulin	TM	82,219

Table 3. Characteristics of some of the growth factor receptors. GIT = gastro-intestinal tract; UT = urogenital tract; TM = transmembrane protein; NKD = no kinase domain present; * = RNA.

prognosis of the patients [174,222]. In normal tissues, c-erbB-2 was found in foetal epithelia of e.g. the human urogenital tract, but in adults its expression was only poor or absent [173].

The c-erbB-3 protein is, like the c-erbB-2 protein, also expressed in tumours, like stomach-, breast-, and colon carcinomas [197,214]. The c-erbB-3 protein is also expressed in hyperplastic mucosa adjacent to gastric carcinomas [214], and in normal adult tissues including the urogenital tract, but is not detectable in haematopoietic tissues [200].

Recently, Plowman et al [196] isolated the cDNA encoding the c-erbB-4 protein.

The tissue distribution of c-erbB-4 is different from that of c-erbB-3 (Table 3) suggesting tissue-specific functions for their ligands, heregulins [43,196].

Activation of the c-erbB-1 to c-erbB-4 receptors results from ligand binding or from heterodimerization of the receptors [43]. These data suggest that all four types of c-erbB proteins have physiological importance. In addition, aberrant expression of c-erbB-1 to c-erbB-3 receptors in tumours is often associated with an increased aggressiveness of the tumour, including TCC.

Three types of high affinity TGFß receptors have been described: type I, type II, and type III. The type I and type II receptors have a cytoplasmic serine/threonine kinase domain [20,68,147]. In contrast, the type III receptor is a proteoglycan, betaglycan, which lacks a signal transduction domain [148,244]. Several studies demonstrated that the absence of the type III receptor did not result in loss of TGFß response, whereas absence of type I receptor did. Hence, it has been suggested that type I and type II receptors require a mutual interaction for mediating the TGFß effects, possibly by dimerization [20,68,139,147]. The type III receptor increases the affinity of the other TGFß receptors for TGFß [149,244]. Recent evidence for functional differences between the type I and type II receptor was given by Wrana et al [251]. The type II receptor, a constitutively active kinase, binds TGFß. The type I receptor in turn binds to the bound TGFß. Upon phosphorylation by the type II receptor, the type I receptor mediates then the signal transduction.

The expression of TGFß receptors is ubiquitous on both normal and neoplastic cells [26,68]. A general correlation between the expression levels of TGFß receptors and the aggressiveness of tumours has not been found.

Four different genes encoding FGF receptors have been identified, and two of these genes generate multiple transcripts by alternative splicing [reviewed in 118]. The binding affinity of the FGFRs differ: both FGFR 1 and FGFR 2 bind FGF-1 and FGF-2 but with different affinities, and FGFR-4 binds FGF-1 better than FGF-2, FGF-4, or FGF-6 [65,182,236,247]. FGF-7 was shown to bind only one splice variant of FGFR 2 [33].

Immunocytochemical analysis by Hughes and Hall [107] showed that in normal adult human tissue FGFR 1 is confined to the microvasculature of many tissues, cardic myocytes, and to the epithelium of lung, cervix, tonsil, and thymus, but not in the urothelium and, in contrast to RNA expression studies, not in kidney.

Low affinity FGF receptors include the transmembrane proteoglycan syndecan, which contains heparan sulphate chains. Syndecan may have a function in embryonal tissue organization [225]. Since the presence of heparin/heparan sulphate chains

enhances the interaction of FGF-2 with the high affinity FGFR [252], syndecan may also serve this function. Its mode of intracellular action is not clear yet since syndecan lacks an intracellular domain for signal transduction.

For PDGF two different receptors have been found: a type α receptor, and a type ß receptor. These receptors dimerize to render homo- or heterodimers, each with specific binding affinity for PDGF (Table 3). Epithelial expression of PDGF type ß receptors has been detected in skin epithelium in vivo only during wound healing [8,10], and in lung, thyroid, gastric, and ovarian carcinomas [9,51,97,98].

A switch in the expression of PDGF type α receptor on normal cultured mesothelial cells to PDGF type ß receptors by malignant mesothelioma cell lines has been observed by Versnel et al [242]. Concomitant with this switch in receptor expression, Gerwin et al [85] and Versnel et al [241] showed that mesothelioma cells expressed strongly the PDGF-B chain mRNA, while normal mesothelial cells expressed predominantly the PDGF-A chain mRNA in vitro. The functional consequences of such a switch in receptor and ligand expression are not clear yet.

The insulin and the type I insulin-like growth factor receptors are structurally and functionally homologous. They consist of 4 subunits, two α and two ß subunits which are linked by disulphide bridges; the greatest amino acid homology resides in the ß chain (approximately 84%). Instead, the type II IGF receptor is a single glycosylated chain without kinase activity and is homologous with the cation-independent mannose-6-phosphate receptor [reviewed in 82,219]. The effects of IGF-I and IGF-II are mostly exerted through the type I IGF receptor. The type II IGF receptor/mannose-6-phosphate receptor regulates the transport of lysosomal enzymes, and regulates a rapid internalization and degradation of IGF-II [reviewed in 82,186,187]. The latter function may serve as a means of IGF-II depletion from the circulation before IGF-II can bind to the type I IGF receptor [189].

In conclusion, the growth factor receptors show a tissue or even cell type specific distribution. However, epithelial cells generally express (one of the) EGF, FGF type 1 or 2, TGFß type I, TGFß type II, and TGFß type III, and type I IGF receptors. PDGF receptors and type II IGF receptors are expressed less frequently on epithelial cells.

Recent studies were performed with transgenic mice in order to define the function of growth factors (or their receptors) in normal tissue and in tumour growth. Transgenic mice that overexpressed TGFα in many tissues, including liver, pancreas,

and mammary glands, frequently showed neoplasia of the liver and the pancreas [reviewed in 56]. The mammary glands showed a high proliferative activity and a very dense network of milk ducts. Muller et al [176] generated FGF-3 transgenic mice. A frequent hyperplasia was found in the prostate and the mammary glands. These and other studies pointed out that an abnormal expression of growth factors, like TGFα and FGF-3, or their receptors may result in abnormal tissue growth, or in tumour growth and invasiveness [reviewed in 1,201]. However, overexpression of growth factor or its receptor alone is not sufficient to cause transformation [56,153].

§ *3.4 Growth factors: Signal transduction pathway*

As pointed out in the previous paragraphs, several growth factors including EGF, PDGF, and insulin/IGFs, exert similar mitogenic effects on different types of cells. This mitogenic effect might be achieved through a similar signal transduction pathway activated by these growth factors. Upon ligand binding, the tyrosine kinase domain of the receptor is activated. Subsequently, many tyrosine-containing proteins that are involved in different signal transduction pathways, are activated by this kinase. Among these are PI(3) kinase, JAK kinases, PLCγ, and Ras proteins [74,108]. Only the role of Ras in the regulation of mitogenesis has recently been elucidated.

Ras p21 is a proto-oncogenic, membrane bound protein. Specific mutations have been found in the encoding *ras* gene in several carcinomas, including approximately 10% of the human TCCs, leading to activation of Ras p21 [32]. Overexpression of (mutated) Ras proteins in TCC cells was shown to enhance invasion of these cells in murine bladder [231]. Other studies showed that also the cytosolic Raf p68-74 protein is involved in mitogen-induced signal transduction [164]. Binding of EGF, PDGF, and insulin to their respective receptors (EGF-R, PDGF-R, and insulin-R) stimulates phosphorylation of tyrosine residues on the receptors, leading to the activation of Ras and Raf, and finally to e.g. activation of transcription factors like myc, jun and fos [reviewed in 15,156]. Ras is a GTP-binding protein and is active only in the GTP-bound conformation. The exchange of GDP for GTP is balanced by GTP-hydrolyzing enzymes (GAP) and guanine nucleotide releasing factors (GRF) or guanine nucleotide dissociation inhibitors (GDI) [reviewed in 30]. Raf is a serine/threonine kinase protein acting downstream from Ras in signal transduction [reviewed in 95].

Recent studies indeed pointed out that EGF, PDGF, and insulin can mediate their effects through a similar signal transduction pathway. These studies also addressed which proteins are involved in the coupling between the receptors, Ras, and Raf. One of the examined GRF's is Sos (Son of Sevenless), a protein homologous to the yeast GRF CDC-25, and associated with the activated Drosophila Sevenless tyrosine kinase

receptor (a photoreceptor) [35]. Another protein involved in the signal pathway is the membrane associated adaptor protein Grb2, a homologue of the yeast Sem5 protein and the Drosophila Drk [190]. Grb2 contains one SH2 (Src homology 2) domain and two SH3 domains. The SH2 domains of different proteins (Src, PLCγ) were found to bind to phosphorylated tyrosine residues in proteins [6]. The SH3 domains bind to proline-rich stretches, also present in Sos. Since activated tyrosine kinase receptors contain phosphorylated tyrosine residues, this suggested an association between receptor, Grb2, Sos, and Ras. The binding of the mammalian homologues of Sos, Grb2, Ras, EGF-R, PDGF-R, and insulin-R have been studied by 1) immunoprecipitations of Sos, Grb2, or EGF-R followed by immunoblotting with anti Grb2 or anti growth factor-R antibodies [17,40,211], 2) binding of Sos and growth factor-R to Grb2-fusionproteins and Grb2-mutant fusionproteins [69], and 3) GTP and GDP exchange kinetics upon overexpression of Sos or Grb2 [81]. In the presence of a mitogen a complex was formed between mitogen-activated GF-R, Grb2, Sos, and Ras [40,211]. For the

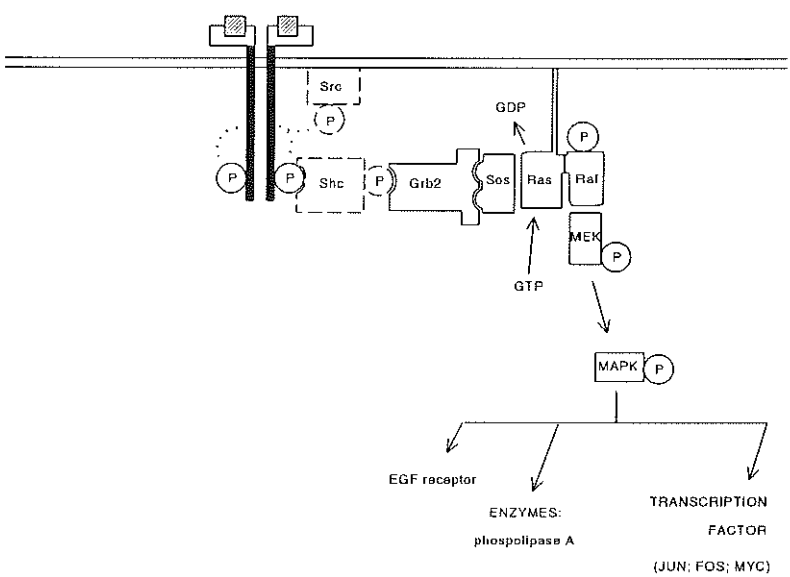

Figure 4. Schematic diagram of interaction between the intracellular tyrosine-phosphorylated residues of growth factor receptors and Ras proteins. The respective proteins in this pathway are described in the text. Some of the final target proteins of the Ras-action are also indicated. Tyrosine-phosphorylated residues of proteins are denoted by an encircled P; Shc and Src do not act in all receptor-Ras activated pathways and are therefore dotted.

activated insulin-R and v-Src, an extra adaptor protein (Shc) was found to be involved in front of Grb2 [69,221].

Other studies revealed a complexing between activated Ras (Ras-GTP)and Raf, but not between inactive Ras and Raf [171,243,245,256]. Associated kinase activity on downstream acting Mitogen Activated Protein Kinase (MAPK) was also detected, suggesting involvement of MAPK kinase in the complex of Ras-Raf [171,232]. Figure 4 depicts schematically the proposed transduction pathway via Ras/Raf and the recently identified proteins involved in the pathway.

The above mentioned mechanism for signal transduction can explain overlapping activities of growth factors. However, different growth factors can also exert each another effect on the same cells (see Table 2 and §3.2). It is known that other transduction pathways exist, already diverging at the cytoplasmatic receptor kinase sites. The specific cellular function of a growth factor is probably determined by those alternative signalling pathways, like activation of phospho-
lipases or PI(3) kinase and subsequent modulations in second messenger signals [141,237]. The specificity of a growth factor is determined by its concentration, the number of receptors per cell, and the activation of which substrates. Alternatively, if a signal transduction pathway is deregulated, e.g. by mutation of a gene encoding a protein that is involved in that pathway, then this cell may not respond to the growth factor that acts through this pathway.

§ 3.5 Growth factors: Current knowledge of growth factors in urothelium

a) Growth factors in normal and neoplastic urothelium.

In urine of normal individuals, human or murine, high levels of up to 20 ng/ml EGF have been measured [42,104]. In contrast to normal individuals, TGFα is present in the urine of patients with bladder carcinoma [129]. EGF was found in superficial urothelial cells of normal individuals, and in human TCC in all cells [142]. The immunohistochemical localization of EGF was similar to that of the EGF receptors on human urothelial cells: EGF receptor proteins are expressed only in normal basal urothelial cells, but in TCC most urothelial cells express EGF receptors. The level of EGF receptor expression in TCC increases generally with higher grades of malignancy [165,181]. These data suggest that both TGFα and EGF are involved in TCC growth, while EGF rather than TGFα is important for the urothelial physiology.

Differences in expression patterns of FGFs have also been found between normal individuals and patients with TCC. Chodak et al [48,49] found in urine from patients with bladder cancer and in their TCC cells an FGF-2 like activity, but not in normal urine or in urothelium. Allen and Maher [4] recently demonstrated FGF-2 and FGF-2 receptor

expression in the human T24 TCC cell line. Ravery et al [204] and Chopin et al [50] demonstrated immunohistochemically FGF-1 expression in human TCC and urine from patients with TCC, but neither FGF-1 expression in normal human urothelium nor FGF-2 expression in normal urothelium or TCC. Other studies showed that the FGF-3 and FGF-4 genes are co-amplified in some bladder carcinomas [230] and the FGF-5 oncogene was first identified in a bladder carcinoma [255]. However, nothing is known about the presence of FGF-3, FGF-4, and FGF-5 proteins in bladder carcinomas. Taken together, these data suggest the involvement of one or more FGFs in TCC growth.

TGFß-like activity has been found in the human urine, regardless of the presence of TCC [129]. In addition, some human TCCs showed a TGFß RNA expression different from normal human urothelium, but no clear correlation could be made between the grade or stage of the TCCs and the TGFß RNA expression. The presence of TGFßs in normal urothelium and urine suggests that TGFß is important for the maintenance and repair of normal urothelium.

A ligand binding study of Iwamura et al [113] suggested the expression of type I IGF receptors on TCC cell lines. Currently, no data are available about IGF actions on urothelial cells.

b) Functional effects of growth factors on urothelial cells.

Several functional studies with human and murine urothelial cells showed that EGF/TGFα and some FGFs modulate the proliferation, or morphology, or migration of these cells in vitro. Messing et al [165] showed that intravesically injected EGF in rat bladder in vivo enhance the [^{3}H]-thymidine uptake in the basal urothelial cells. In vitro studies showed that EGF stimulate the proliferation of human TCC cell lines and murine normal and neoplastic urothelial cell lines [137, 166,169,254]. Both EGF and TGFα induce some morphological characteristics of neoplasia in primary bladder explant cultures, like elongation of cells, blebbing, and loss of polarity [134].

FGF-1 and FGF-2 induce the proliferation of NBT-II, a rat bladder carcinoma cell line [238]. Furthermore, both FGF-1 and TGFα, but not FGF-2, induce the migration of the NBT-II cells in vitro [83,124], and FGF-1, FGF-4, and TGFα induce NBT-II cells to invade into primary rat bladder explants in vitro [233]. However, virtually nothing is known about functional effects of FGFs on normal urothelium, neither in vitro nor in vivo. Also, the functional role of TGFß on urothelial cells has not been elucidated. In analogy to the observed effects of TGFßs on other epithelia (see above), TGFß might also be capable of modulating proliferation and differentiation of urothelial cells.

Increasing evidence is now emerging on expression of PDGF and its receptors in epithelial cells under certain conditions, i.e. wound healing, cystitis, or in carcinomas

(see §3). Both renal glomerular and tubular epithelial cells as well as the T24 TCC cells can express PDGF-B mRNA [5,34,77]. In addition, PDGF-B protein levels are higher in urine of patients with bladder cancer [188]. However, data about the direct actions of PDGFs on any epithelial cell are sparse (see §3), and nothing is known yet about the expression of PDGFs or their receptors in normal urothelial cells.

§ 4 *Aim of the thesis*

Many studies predominantly on skin epithelial cells indicated that EGF, TGFα, FGF-1, FGF-2, TGFß, PDGF-AA, and PDGF-BB have specific functions e.g. during epidermal wound healing or in tumour growth. Studies on the expression of growth factors and their receptors in normal urothelium or in TCC suggested that growth factors are also implicated in the maintenance of the urothelium and in the growth of TCC. However, the specific function of growth factors in the maintenance and repair of normal urothelium, or the abnormal growth of urothelial cells in neoplasia has not yet been elucidated. The studies reported in this thesis focus on the effects of growth factors on normal, regenerating, and neoplastic mouse urothelium.
Several specific questions were addressed:

1) which growth factors modulate the proliferation, differentiation, migration, and apoptosis, in normal urothelial cells?
2) do cultures of normal urothelial cells that reflect intact urothelium, respond differently to growth factors as compared with cultures that reflect regenerating urothelium?
3) do the in vitro observed effects of growth factors with in vivo expression of these growth factors and their receptors during wound healing?.
4) do normal and neoplastic urothelial cells in vitro differ in their response to growth factors?
5) can TCC cells in vivo affect the growth of surrounding urothelium by paracrine action of TCC-derived growth factors?

Studies on these functions may provide a basis for therapeutic experiments in which injured bladder epithelium can be treated with specific growth factors. These studies may also provide a basis for explaining differences in the behaviour of TCC compared with normal urothelium.

§ 5 *REFERENCES*

1 Aaronson AS: Growth factors and cancer. Science 254, 1991:1146-1153.

2 Abraham JA, Mergia A, Whang JL, Tumolo A, Friedman J, Hjerrild KA, Gospodarowicz D, Fiddes JC: Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science 233, 1986:545-548.

3 Albiston AL, Taylor RG, Herington AC, Beveridge DJ, Fuller PJ: Divergent ileal IGF-I and IGFBP-3 gene expression after small bowel resection: a novel mechanism to amplify IGF action ? Mol. Cell. Endocrinol. 83, 1992:R17-R20.

4 Allen LE, Maher PA: Expression of basic fibroblast growth factor and its receptor in an invasive bladder carcinoma cell line. J. Cell. Physiol. 155, 1993:368-375.

5 Alpers CE, Seifert RA, Hudkins KL, Johnson RJ, Bowen-Pope DF: PDGF-receptor localizes to mesangial, parietal epithelial, and interstitial cells in human and primate kidneys. Kidney Int. 43, 1993:286-294.

6 Anderson D, Koch CA, Grey L, Ellis C, Moran MF, Pawson T: Binding of SH2 domains of phospholipase $C_{\gamma}1$, GAP, and Src to activated growth factor receptors. Science 250, 1990:979-981.

7 Ando Y, Jensen PJ: Epidermal growth factor and insulin-like growth factor I enhance keratinocyte migration. J. Invest. Dermatol. 100, 1993:633-639.

8 Antoniades HN, Galanopoulois T, Neville-Golden J, Kiritsy CP, Lynch SE: Injury induces *in vivo* expression of platelet-derived growth factor (PDGF) and PDGF receptor mRNAs in skin epithelial cells and PDGF mRNA in connective tissue fibroblasts. Proc. Natl. Acad. Sci. USA 88, 1991:565-569.

9 Antoniades HN, Galanopoulos T, Neville-Golden J, O'Hara CJ: Malignant epithelial cells in primary human lung carcinomas coexpress *in vivo* platelet-derived growth factor (PDGF) and PDGF receptor mRNAs and their protein products. Proc. Natl. Acad. Sci. USA 89, 1992:3942-3946.

10 Antoniades HN, Galanopoulos T, Neville-Golden J, Kiritsy CP, Lynch SE: Expression of growth factor and receptor mRNAs in skin epithelial cells following acute cutaneous injury. Am. J. Pathol. 142, 1993:1099-1109.

11 Anzano MA, Rieman D, Prichett W, Bowen-Pope DF, Greig R: Growth factor production by human colon carcinoma cell lines. Cancer Res. 49, 1989:2898-2904.

12 Aronson M, Medalia O, Amichay D, Nativ O: Endotoxin-induced shedding of viable uroepithelial cells is an antimicrobial defense mechanism. Infect. Immun. 56, 1988:1615-1617.

13 Asami O, Ihara I, Shimidzu N, Shimizu S, Tomita Y, Ichihara A, Nakamura T: Purification and characterization of hepatocyte growth factor from injured liver of carbon tetrachloride-treated rats. J. Biochem. 109, 1991:8-13.

14 Assoian RK, Frolik CA, Roberts AB, Miller DM, Sporn MB: Transforming growth factor-ß

controls receptor levels for epidermal growth factor in NRK fibroblasts. Cell 36, 1984:35-41.

15 Avruch J, Zhang X-F, Kyriakis JM: Raf meets Ras: completing the framework of a signal transduction pathway. Trends Biochem. Sci. 19, 1994:279-283.

16 Ayres PH, Shinohara Y, Frith CH: Morphological observations on the epithelium of the developing urinary bladder of the mouse and rat. J. Urol. 133, 1985:506-512.

17 Baltensperger K, Kozma LM, Cherniack AD, Klarlund JK, Chawla A, Banerjee U, Czech MP: Binding of the Ras activator Son of sevenless to insulin receptor substrate-1 signaling complexes. Science 260, 1993:1950-1952.

18 Barrandon Y, Green H: Cell migration is essential for sustained growth of keratinocyte colonies: the roles of transforming growth factor-α and epidermal growth factor. Cell 50, 1987:1131-1137.

19 Barreca A, De Luca M, Del Monte P, Bondanza S, Damonte G, Cariola G, Di Marco E, Giordano G, Cancedda R, Minuto F: In vitro paracrine regulation of human keratinocyte growth by fibroblast-derived insulin-like growth factors. J. Cell. Physiol. 151, 1992:262-268.

20 Bassing CH, Yingling, JM, Howe, DJ, Wang, T, Wu He W, Gustafson ML, Shah P, Donahoe PK, Wang X-F: A transforming growth factor ß type I receptor that signals to activate gene expression. Science 263, 1994:87-89.

21 Bassols A, Massagué J: Transforming growth factor ß modulates the expression and structure of extracellular matrix chondroitin/dermatan sulfate proteoglycans. J. Biol. Chem. 263, 1988:3039-3045.

22 Bates B, Hardin J, Zhan X, Drickamer K, Goldfarb M: Biosynthesis of human fibroblast growth factor-5. Mol. Cell. Biol. 11, 1991:1840-1845.

23 Battegay EJ, Raines EW, Seifert RA, Bowen-Pope DF, Ross R: TGF-ß induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell 63, 1990:515-524.

24 Bellot F, Moolenaar W, Kris R, Mirakhur B, Verlaan I, Ullrich A, Schlessinger J, Felder S: High-affinity epidermal growth factor binding is specifically reduced by a monoclonal antibody, and appears necessary for early responses. J. Cell Biol. 110, 1990:491-502.

25 Benharroch D, and Birnbaum D: Biology of the fibroblast growth factor gene family. Isr. J. Med. Sci. 26, 1990:212-219.

26 Bennett NT, Schultz GS: Growth factors and wound healing: biochemical properties of growth factors and their receptors. Am. J. Surg. 165, 1993:728-737.

27 Betsholtz C, Bergh J, Bywater M, Pettersson M, Johnsson A, Heldin C-H, Ohlsson R, Knott TJ, Scott J, Bell GI, Westermark B: Expression of multiple growth factors in a human lung cancer cell line. Int. J. Cancer 39, 1987:502-507.

28 De Boer WI, Rebel JMJ, Foekens JA, Vermey M, Van der Kwast TH: Characterization of mouse urothelial cell lines in different phases of transitional-cell carcinogenesis. Int. J. Cancer

54, 1993:1022-1027.

29 Boes M, Dake BL, Bar RS: Interactions of cultured endothelial cells with TGF-ß, bFGF, PDGF and IGF-I. Life Sciences 48, 1991:811-821.

30 Boguski MS, McCormick F: Proteins regulating Ras and its relatives. Nature 366, 1993:643-654.

31 Bolander ME: Regulation of fracture repair by growth factors. Proc. Soc. Exp. Biol. Med. 200, 1992:165-170.

32 Bos JL: *ras* Oncogenes in human cancer: a review. Cancer Res. 49, 1989:4682-4689.

33 Bottaro DP, Rubin JS, Ron D, Finch PW, Florio C, Aaronson SA: Characterization of the receptor for keratinocyte growth factor. J. Biol. Chem. 265, 1990:12767-12770.

34 Bowen-Pope DF, Vogel A, Ross R: Production of platelet-derived growth factor-like molecules and reduced expression of platelet-derived growth factor receptors accompany transformation by a wide spectrum of agents. Proc. Natl. Acad. Sci. USA 81, 1984:2396-2400.

35 Bowtell D, Fu P, Simon M, Senior P: Identification of murine homologues of the *Drosophila* Son of sevenless gene: potential activators of ras. Proc. Natl. Acad. Sci. USA 89, 1992:6511-6515.

36 Boyland E, Wallace DM, Williams DC: Activity of the enzymes sulfatase and ß-glucuronidase in the urine, serum and bladder tissue. Br. J. Cancer 9, 1955:62-79.

37 Brewitt B, Clark JI: Growth and transparency in the lens, an epithelial tissue, stimulated by pulses of PDGF. Science 242, 1988:777-779.

38 Brown GL, Curtsinger L, Brightwell JR, Ackerman DM, Tobin GR, Polk HC, George-Nascimento G, Valenzuela P, Schultz GS: Enhancement of epidermal regeneration by biosynthetic epidermal growth factor. J. Exp. Med. 163, 1986:1319-1324.

39 Bryant-Greenwood GD: Relaxin as a new hormone. Endocrine Rev. 3, 1982:62-90.

40 Buday L, Downward J: Epidermal growth factor regulates $p21^{ras}$ through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 73, 1993:611-620.

41 Campochiaro PA, Glaser BM: Platelet-derived growth factor is chemotactic for human retinal pigment epithelial cells. Arch. Ophthalmol. 103, 1985:576-579.

42 Carpenter G: Epidermal growth factor: biology and receptor metabolism. J. Cell. Sci. Suppl. 3, 1985:1-9.

43 Carraway III KL, Cantley LC: A Neu acquitance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. Cell 78, 1994:5-8.

44 Cate RL, Matteliano RJ, Hession C, Tizard R, Farber NM, Cheung A, Ninfa EG, Frey AZ,

Gash DJ, Chow EP, Fisher RA, Bertonis JM, Torres G, Wallner BP, Ramachandranm KL, Manganaro TF, MacLaughlin DT, Donahow PK: Isolation of the bovine and human genes for Müllerian inhibitory substance and expression the human gene in animal cells. Cell 45, 1986:685-698.

45 Chan CP, Krebs EG: Epidermal growth factor stimulates glycogen synthase activity in cultured cells. Proc. Natl. Acad. Sci. USA 82, 1985:4563-4567.

46 Chen Y, Rabinovitch PS: Platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor I regulate specific cell-cycle parameters of human diploid fibroblasts in serum-free culture. J. Cell. Physiol. 140, 1989:59-67.

47 Cheng K, Larner J: Intracellular mediators of insulin action. Ann. Rev. Physiol. 47, 1985:405-424.

48 Chodak GW, Shing Y, Borge M, Judge SM, Klagsbrun M: Presence of heparin binding growth factor in mouse bladder tumors and urine from mice with bladder cancer. Cancer Res. 46, 1986:5507-5510.

49 Chodak GW, Hospelhorn V, Judge SM, MAyforth R, Koeppen H, Sasse J: Increased levels of fibroblast growth factor-like activity in urine from patients with bladder or kidney cancer. Cancer Res. 48, 1988:2083-2088.

50 Chopin DK, Caruelle J-P, Colombel M, Palcy S, Ravery V, Caruelle D, Abbou CC, Barritault D: Increased immunodetection of acidic fibroblast growth factor in bladder cancer, detectable in urine. J. Urol. 150, 1993:1126-1130.

51 Chung CK, Antoniades HN: Expression of c-*sis*/platelet-derived growth factor B, insulin-like growth factor I, and transforming growth factor alpha messenger RNAs and their respective receptor messenger RNAs in primary human gastric carcinomas: in vivo studies with in situ hybridization and immunocytochemistry. Cancer Res. 52, 1992:3453-3459.

52 Clemmons DR, Van Wyk JJ: Somatomedin-C and platelet-derived growth factor stimulate human fibroblast replication. J. Cell. Physiol. 106, 1981:361-367.

53 Clemmons DR: IGF binding proteins: regulation of cellular actions. Growth Regul. 2, 1992:80-87.

54 Clowes AW, Cherry RJ, Chapman D: Physical properties of lecithin-cerebroside bilayers. Biochim. Biophys. Acta 249, 1971:301-317.

55 Coffey RJ, Derynck R, Wilcox JN, Bringman TS, Goustin AS, Moses HL, Pittelkow MR: Production and auto-induction of transforming growth factor-α in human keratinocytes. Nature 328, 1987:817-820.

56 Coffey RJ, McCutchen CM, Graves-Deal R, Polk WH: Transforming growth factors and related peptides in gastrointestinal neoplasia. J. Cell. Biochem. Suppl. 16G, 1992:111-118.

57 Cohen SM: Toxic and nontoxic changes induced in the urothelium by xenobiotics. Toxicol. Applied Pharmacol. 101, 1989:484-498.

58 Cordon-Cardo C, Vlodavsky I, Haimovitz-Friedman A, Hicklin D, Fuks Z: Expression of basic fiboblast growth factor in normal human tissues. Lab. Invest. 63, 1990:832-840.

59 Cullen KJ, Smith HS, Hill S, Rosen N, Lippman ME: Growth factor messenger RNA expression by human breast fibroblasts from benign and malignant lesions. Cancer Res. 51, 1991:4978-4985.

60 Davis CP, Cohen MS, Gruber MB, Anderson MD, Warren MM: Urothelial hyperplasia and neoplasia: a response to chronic urinary tract infections in rats. J. Urol. 132, 1984:1025-1031.

61 DeLapp NW, Dieckman Dk: Effect of basic fibroblast growth factor (bFGF) and insulin-like growth factor type I (IGF-I) and type II (IGF-II) on adult human keratinocyte growth and fibronectin secretion. J. Invest. Dermatol. 94, 1990:777-780.

62 Delli-Bovi P, Curatola AM, Newman KM, Sato Y, Moscatelli D, Hewick RM, Rifkin DB, Basilico C: Processing, secretion, and biological properties of a novel growth factor of the fibroblast growth factor family with oncogenic potential. Mol. Cell. Biol. 8, 1988:2933-2941.

63 Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV: Human transforming growth factor-α: precursor structure and expression in E. coli. Cell 38, 1984:287-297.

64 Derynck R: Transforming growth factor α. Cell 54, 1988:593-595.

65 Dionne CA, Crumley G, Bellot F, Kaplow JM, Searfoss G, Ruta M, Burgess WH, Jaye M, Schlessinger J: Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. EMBO J. 9, 1990:2685-2692.

66 Dixon M, Deed R, Acland P, Moore R, Whyte A, Peters G, Dickson C: Detection and characterization of the fibroblast growth factor-related oncoprotein INT-2. Mol. Cell. Biol. 9, 1989:4896-4902.

67 Drop SLS, Schuller AGP, Lindenbergh-Kortleve DJ, Groffen C, Brinkman A, Zwarthoff EC: Structural aspects of the IGFBP family. Growth Regul. 2, 1992:69-79.

68 Ebner R, Chen R-H, Shum L, Lawler S, Zioncheck TF, Lee A, Lopez AR, Derynck R: Cloning of a type I TGF-ß receptor and its effect on TGF-ß binding to the type II receptor. Science 260, 1993:1344-1348.

69 Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA: Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature 363, 1993:45-51.

70 Elliott B, Östman A, Westermark B, Rubin K: Modulation of growth factor responsiveness of murine mammary carcinoma cells by cell matrix interactions: correlation of cell proliferation and spreading. J. Cell. Physiol. 152, 1992:292-301.

71 Esienger M: Epidermal derived factors. Presented at the 3rd International Wound Healing Symposium, Miami, 1990.

72 Fager G, Camejo G, Bondjers G: Heparin-like glycosaminoglycans influence growth and

phenotype of human arterial smooth muscle cells in vitro. I. Evidence for reversible binding and inactivation of the platelet-derived growth factor by heparin. In Vitro Cell. Dev. Biol. 28A, 1992:168-175.

73 Federspiel SJ, DiMari SJ, Howe AM, Guerry-Force ML, Haralson MA: Extracellular matrix biosynthesis by cultered fetal rat lung epithelial cells. III. Effects of chronic exposure to epidermal growth factor on growth, differentiation, and collagen biosynthesis. Lab. Invest. 64, 1991:463-473.

74 Feig LA, Schaffhausen B: The hunt for Ras targets. Nature 370, 1994:508-509.

75 Finch PW, Rubin JS, Miki T, Ron D, Aaronson AS: Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. Science 245, 1989:752-755.

76 Firth JA, Hicks RM: Membrane specialization and synchronized cell death in developing rat transitional epithelium. J. Anat. 113, 1972:95-107.

77 Floege J, Johnson RJ, Alpers CE, Fatemi-Nainie S, Richardson CA, Gordon K, Couser WG: Visceral glomerular epithelial cells can proliferate *in vivo* and synthesize platelet-derived growth factor B-chain. Am. J. Pathol. 142, 1993:637-650.

78 Florini JR, Ewton DZ: Induction of gene expression in muscle by the IGFs. Growth Regul. 2, 1992:23-29.

79 Froesch ER, Schmid C, Schwander J, Zapf J: Actions of insulin-like growth factors. Ann. Rev. Physiol. 47, 1985:443-467.

80 Fuller-Pace F, Peters G, Dickson C: Cell transformation by kFGF requires secretion but not glycosylation. J. Cell Biol. 115, 1991:547-555.

81 Gale NW, Kaplan S, Lowenstein EJ, Schlessinger J, Bar-Sagi D: Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. Nature 363, 1993:88-92.

82 Gammeltoft S: Insulin-like growth factors and insulin: gene expression, receptors and biological actions. In: Martinez ed.:Peptide hormones as prohormones. Ellis Harwood Ltd., 1989.

83 Gavrilovic J, Moens G, Thierry JP, Jouanneau J: Expression of transfected transforming growth factor α induces a motile fibroblast-like phenotype with extracellular matrix-degrading potential in a rat bladder carcinoma cell line. Cell Regul. 1, 1990:1003-1014.

84 Gelfand M, Weinberg RW, Castle WM: Relation between carcinoma of the bladder and infestation with *Schistosoma hematobium*. Lancet 1, 1967:1249-1251.

85 Gerwin BI, Lechner JF, Reddel RR, Roberts AB, Robbins KC, Gabrielson EW, Harris CC: Comparison of production of transforming growth factor-ß and platelet-derived growth factor by normal human mesothelial cells and mesothelioma cell lines. Cancer Res. 47, 1987:6180-6184.

86 Glick AB, Danielpour D, Morgan D, Sporn MB, Yuspa SH: Induction and autocrine receptor binding of transforming growth factor-ß2 during terminal differentiation of primary

mouse keratinocytes. Mol. Endocrinol. 4, 1990:46-52.

87 Grotendorst GR, Chang T, Seppä HE, Kleinman HK, Martin GR: Platelet-derived growth factor is a chemoattractant for vascular smooth muscle cells. J. Cell. Physiol. 113, 1982:261-266.

88 Han JK, Martin GR: Embryonic expression of Fgf-6 is restricted to the skeletal muscle lineage. Dev. Biol. 158, 1993:549-554.

89 Harpel JG, Metz CN, Kojima S, Rifkin DB: Control of transforming growth factor-ß activity: latency vs. activation. Progr. Growth Factor Res. 4, 1992:321-335.

90 Harris RC, Daniel TO: Epidermal growth factor binding, stimulation of phosphorylation, and inhibition of gluconeogenesis in rat proximal tubule. J. Cell. Physiol. 139, 1989:383-391.

91 Hart CE, Bowen-Pope DF: Platelet-derived growth factor receptor: current views of the two-subunit model. J. Invest. Dermatol. 94, 1990:53S-57S.

92 Haub M, Drucker B, Goldfarb M: Expression of the murine fibroblast growth factor 5 gene in the adult central nervous system. Proc. Natl. Acad. Sci. 87, 1990:8022-8026.

93 Hebda PA. Stimulatory effects of transforming growth factor-beta and epidermal growth factor on epidermal cell outgrowth from porcine skin explant cultures. J. Invest. Dermatol. 91. 1988:440-445.

94 Hébert JM, Basilico C, Goldfarb M, Haub O, Martin GR: Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression patterns during embryogenesis. Dev. Biol. 138, 1990:454-463.

95 Heidecker G, Kölch W, Morrison DK, Rapp UR: The role of Raf-1 phosphorylation in signal transduction. Adv. Cancer Res. 58, 1992:53-73.

96 Heldin C-H, Betsholtz C, Johnsson A, Nister M, Ek B, Ronnstrand L, Wasteson A, Westermark B: Platelet-derived growth factor: mechanism of action and relation to oncogenes. J. Cell Sci. Suppl. 3, 1985:65-76.

97 Heldin N-E, Cvejic D, Smeds S, Westermark B: Coexpression of functionally active receptors for thyrotropin and platelet-derived growth factor in human thyroid carcinoma cells. Endocrinology 129, 1991:2187-2193.

98 Henriksen R, Funa K, Wilander E, Bäckström T, Ridderheim M, Öberg K: Expression and prognostic significance of platelet-derived growth factor and its receptors in epithelial ovarian neoplasms. Cancer Res. 53, 1993:4550-4554.

99 Hernandez J, Zarnegar R, Michalopoulos GK: Characterization of the effects of human placental HGF on rat hepatocytes. J. Cell. Physiol. 150, 1992:116-121.

100 Hicks RM, Kettere B, Warren RC: The ultrastructure and chemistry of the luminal plasma membrane of the mammalian urinary bladder: a structure with low permeability to water and ions. Philos. Trans. R. Soc. B 268, 1974:23-38.

101 Hicks RM: The mammalian urinary bladder: an accommodating organ. Biol. Rev. 50, 1975:215-246.

102 Higashiyama S, Abraham JA, Klagsbrun M: Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration. J. Cell Biol. 122, 1993:933-940.

103 Hiraki Y, Rosen OM, Birnbaum MJ: Growth factors rapidly induce expression of the glucose transporter gene. J. Biol. Chem. 263, 1988:13655-13622.

104 Hirata Y, Orth DN: Concentrations of epidermal growth factor, nerve growth factor, and submandibular gland renin in male and female mouse tissue and fluids. Endocrinology 105, 1979:1383-1387.

105 Holly JMP, Wass JAH: Insulin-like growth factors; autocrine, paracrine or endocrine ? New perspectives of the somatomedin hypothesis in the light of recent developments. J. Endocrinol. 122, 1989:611-618.

106 Hori A, Sasada R, Matsutani E, Naito K, Sakura Y, Fujita T, Kozai Y: Suppression of solid tumor growth by immunoneutralizing monoclonal antibody against human basic fiboblast growth factor. Cancer Res. 51, 1991:6180-6184.

107 Hughes SE, Hall PA: Immunolocalization of fibroblast growth factor receptor 1 and its ligands in human tissues. Lab. Invest. 69, 1993:173-182.

108 Ihle JN, Witthuhn BA, Quelle FW, Yamamoto K, Thierfelder WE, Kreider B, Silvenoinen O: Signaling by the cytokine receptor superfamily: JAKs and STATs. Trends Biochem. Sci. 19, 1994:222-227.

109 Inoue H, Kato Y, Iwamoto M, Hiraki Y, Sakuda M, Suzuki F: Stimulation of cartilage-matrix proteoglycan synthesis by morphologically transformed chondrocytes grown in the presence of fibroblast growth factor and transforming growth factor-beta. J. Cell. Physiol. 138, 1989:329-337.

110 Isaacs HV, Tannahill D, Slack JM: Expression of a novel FGF in the Xenopus embryo. A new candidate inducing factor for mesoderm formation and anteroposterior specification. Development 114, 1992:711-720.

111 Ishihara M, Fedarko NS, Conrad HE: Involvement of phosphatidylinositol and insulin in the coordinate regulation of proteoheparan sulfate metabolism and hepatocyte growth. J. Biol. Chem. 262, 1987:4708-4716.

112 Ishikawa M, Takayanagi Y, Sasaki K: Modification of cyclophosphamide-induced urotoxicity by buthionine sulfoximine and disulfiram in mice. Res. Commun. Chem. Pathol. Pharmacol. 65, 1989:265-268.

113 Iwamura M, Ishibe M, Sluss PM, Cockett ATK: Charaterization of insulin-like growth factor I binding sites in human bladder cancer cell lines. Urol. Res. 21, 1993:27-32.

114 Jakobovits A, Shackleford GM, Varmus HE, Martin GR: Two proto-oncogenes implicated in mammary carcinogenesis, *int-1* and *int-2*, are independently regulated during mouse development. Proc. Natl. Acad. Sci. USA 83, 1986:7806-7810.

115 Jakowlew SB, Dillard PJ, Kondaiah P, Sporn MB, Roberts AB: Complementary deoxyribonucleic acid cloning of a novel transforming growth factor ß messenger ribonucleic acid from chick embryo chondrocytes. Mol. Endocrinol. 2, 1988:747-755.

116 Jaye M, Howk R, Burgess W, Ricca GA, Chiu I-U, Ravera MW, O'Brien SJ, Modi WS, Maciag T, Drohan WN: Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosome localization. Science 233, 1986:541-545.

117 Jhappan C, Stahle C, Harkins RN, Fausto N, Smith GH, Merlino GT: TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. Cell 61, 1990:1137-1146.

118 Johnson DE, Williams LT: Structural and functional diversity in the FGF receptor multigene family. Adv. cancer Res. 60, 1993:1-41.

119 Jones RF, Debiec-Rychter M, Wang CY: Chemical carcinogenesis of the urinary bladder — a status report. J. Cancer Res. Clin. Oncol. 118, 1992:411-419.

120 De Jong WH, Steerenberg PA, Ruitenberg EJ: *Bacillus Calmette-Guérin* (BCG) and its use for cancer immunotherapy. In: Tumor immunology - mechanisms, diagnosis, therapy. Den Otter W, and Ruitenberg EJ, eds. Elsevier Science
Publishers, pp 283-307.

121 Josephs SF, Ratner L, Clarke MF, Westin EH, Reitz MS, Wong-Staal F: Transforming potential of human c-*sis* nucleotide sequences encoding platelet-derived growth factor. Science 225, 1984:636-639.

122 Josephy PD: Activation of aromatic amines by prostaglandin H synthase. Free Radical Biol. Med. 6, 1989:533-540.

123 Jost SP: Cell cycle of normal bladder urothelium in developing and adult mice. Virchows Archiv B Cell Pathol. 57, 1989:27-36.

124 Jouanneau J, Gavrilovic J, Caruelle D, Jaye M, Moens G, Caruelle J-P, Thierry J-P: Secreted or nonsecreted forms of acidic fibroblast growth factor produced by transfected epithelial cells influence cell morphology, motility, and invasive potential. Proc. Natl. Acad. Sci. USA 88, 1991:2893-2897

125 Ju WD, Velu TJ, Vass WC, Papageorge AG, Lowy DR: Tumorigenic transformation of NIH 3T3 cells by the autocrine synthesis of transforming growth factor α. New Biol. 3, 1991:380-388.

126 Kamalati T, Howard M, Brooks RF: IGF-I induces differentiation in a transformed human keratinocyte line. Development 106, 1989:283-293.

127 Kandel J, Bossy-Wetzel E, Radvanyi F, Klagsbrun M, Folkman J, Hanahan
D. Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. Cell 66, 1991:1095-1104.

128 Ketterer B, Hicks RM, Christodoulides L, Beale D: Studies of the chemistry of the luminal plasma membrane of rat bladder epithelial cells. Biochim. Biophys. Acta 311, 1973:180-190.

129 Kimball ES, Bohn WH, Cockley KD, Warren TC, Sherwin SA: Distinct high-performance liquid chromatography pattern of transforming growth factor activity in urine of cancer patients with that of normal individuals. Cancer Res. 44, 1984:3616-3619.

130 Kimelman D, Christian JL, Moon RT: Synergistic principles of development: overlapping patterning systems in Xenopus mesoderm induction. Development 116, 1992:1-9.

131 Kingsley DM: The TGF-ß superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes and Dev. 8, 1994:133-146.

132 Klagsbrun M: The fibroblast growth factor family: structural and biological properties. Progr. Growth Factor Res. 1, 1989:207-235.

133 Knighton DR, Phillips GD, Fiegel VD: Wound healing angiogenesis: indirect stimulation by basic fibroblast growth factor. J. Trauma 30, 1990:S134-S144.

134 Knowles MA, Hicks RM, Dave H, Harvey AE, Roberts AB, Sporn MB: Transforming growth factors induce markers of neoplasia in cultured adult rat bladder. Carcinogenesis 6, 1985:595-604.

135 Kratz G, Lake M, Ljungström K, Forsberg G, Hægerstrand A, Gidlund M: Effect of recombinant IGF binding protein-1 on primary cultures of human keratinocytes and fibroblasts: selective enhancement of IGF-1 but not IGF-2-induced cell proliferation. Exp. Cell Res. 202, 1992:381-385.

136 Kudlow JE, Cheung C-YM, Bjorge JD: Epidermal growth factor stimulates the synthesis of its own receptor in a human breast cancer cell line. J. Biol. Chem. 261, 1986:4134-4138.

137 Kuranami M, Yamaguchi K, Fuchigami M, Imanishi K, Watanabe T, Abe K, Asanuma F, Hiki Y: Effect of urine on clonal growth of human bladder cancer cell lines. Cancer Res. 51, 1991:4631-4635.

138 Kurokowa M, Lynch K, Podolsky DK: Effects of growth factors on an intestinal epithelial cell line: transforming growth factor ß inhibits proliferation and stimulates differentiation. Biochem. Biophys. Res. Commun. 142, 1987:775-782.

139 Laiho M, Weis FMB, Massagué J: Concommitant loss of transforming growth factor (TGF)-ß receptor types I and II in TGF-ß resistant cell mutants implicates both receptor types in signaltransduction. J. Biol. Chem. 265, 1990:18518-18524.

140 Lahm H, Odartchenko N: Role of transforming growth factor ß in colorectal cancer. Growth Factors 9, 1993:1-9.

141 Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL: A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260, 1993:315-319.

142 Lau JLT, Fowler JE, Ghosh L: Epidermal growth factor in the normal and neoplastic kidney and bladder. J. Urol. 139, 1988:170-175.

143 Lee K, Ewing JF, Sluss PM: Effect of bacterial lipopolysaccharide on growth of murine

bladder cancer, MBT-2. Urol. Res. 17, 1989:285-288.

144 Lee K-H, Bowen-Pope DF, Reed RR: Isolation and characterization of the α platelet-derived growth factor receptor from rat olfactory epithelium. Mol. Cell. Biol. 10, 1990:2237-2246.

145 Levine LA, Richie JP: Urological complications of cyclophosphamide. J. Urol. 141, 1989:1063-1069.

146 Li JJ, Huang YQ, Moscatelli D, Nicolaides A, Zhang WC, Friedman-Kien AE: Expression of fibroblast growth factors and their receptors in acquired immunodeficiency syndrome-associated Kaposi sarcoma tissue and derived cells. Cancer 72, 1993:2253-2259.

147 Lin HY, Wang XF, Ng-Eaton E, Weinberg RA, Lodish HF: Expression cloning of the TGFß type II receptor, a functional transmembrane serine/threonine kinase. Cell 68, 1992:775-785.

148 López-Casillas F, Cheifetz S, Doody J, Andres JL, Lane WS, Massagué J: Structure and function of the membrane proteoglycan betaglycan, a component of the TGFß receptor system. Cell 67, 1991:785-795.

149 López-Casillas F, Wrana JL, Massagué J: Betaglycan presents ligand to the TGFß signaling receptor. Cell 73, 1993:1435-1444.

150 Lu K, Campisi J: Ras proteins are essential and selective for the action of insulin-like growth factor 1 late in the G_1 phase of the cell cycle in BALB/c murine fibroblasts. Proc. Natl. Acad. Sci. USA 89, 1992:3889-3893.

151 Lynch SE, Colvin RB, Antoniades HN: Growth factors in wound healing. Single and synergistic effects on partial thickness porcine skin wounds. J. Clin. Invest. 84, 1989:640-646.

152 Maher PA: Modulation of the epidermal growth factor receptor by basic fibroblast growth factor. J. cell. Physiol. 154, 1993:350-358.

153 Di Marco E, Pierce JH, Fleming TP, Kraus MH, Molloy CJ, Aaronson AS, Di Fiore PP: Autocrine interaction between TGFα and the EGF-receptor: Quantitative requirements for induction of the malignant phenotype. Oncogene 4, 1989:831-838.

154 Marics I, Adelaide J, Raybaud F, Mattei M-G, Coulier F, Planche J, de Lapeyriere O, Birnbaum D: Characterization of the HST-related FGF.6 gene, a new member of the fibroblast growth factor gene family. Oncogene 4, 1989:335-340.

155 Martin P, Hopkinson-Wooley J, McCluskey J: Growth factors and cutaneous wound repair. Progress Growth Factor Res. 4, 1992:25-44.

156 Marx J: Forging a path to the nucleus. Science 260, 1993:1588-1590.

157 Massagué J: Transforming growth factor-ß modulates the high-affinity receptors for epidermal growth factor and transforming growth factor-α. J. Cell Biol. 100, 1985:1508-1514.

158 Massagué J, Cheifetz S, Endo T, Nadal-Ginard B: Type ß transforming growth factor is an inhibitor of myogenic differentiation. Proc. Natl. Acad. Sci. USA 83, 1986:8206-8210.

159 Massagué J, Attisano L, Wrana JL: The TGF-ß family and its composite receptors. Trends Cell Biol. 4, 1994:172-178.

160 Matejka GL, Jennische E: IGF-I binding and IGF-I mRNA expression in the post-ischemic regenerating rat kidney. Kidney Int. 42, 1992:1113-1123.

161 McCredie M, Stewart JH: Does paracetamol cause urothelial cancer or renal papillary necrosis ? Nephron 49, 1988:296-300.

162 McNeil PL, Muthukrishnan L, Warder E, D'Amore PA: Growth factors are released by mechanically wounded endothelial cells. J. Cell Biol. 109, 1989:811-822.

163 McQueen CA, Way BM, Williams GM: Biotransformation of aromatic amines to DNA-damaging products by urinary bladder organ cultures. Carcinogenesis 8, 1987:401-404.

164 Medema RH, De Vries-Smits AMM, Van der Zon GCM, Maassen JA, Bos JL: Ras activation by insulin and epidermal growth factor through enhanced exchange of guanine nucleotides on p21*ras*. Mol. Cell. Biol. 13, 1993:155-162.

165 Messing EM, Hanson P, Ulrich P, Erturk E: Epidermal growth factor interactions with normal and malignant urothelium: *in vivo* and *in situ* studies. J. Urol. 138, 1987:1329-1335.

166 Messing EM, Reznikoff CA: Normal and malignant human urothelium: *in vitro* effects of epidermal growth factor. Cancer Res. 47, 1987:2230-2235.

167 Miyamoto M, Naruo K-I, Seko C, Matsumoto S, Kondo T, Kurokawa T: Molecular cloning of a novel cytokine cDNA encoding the ninth member of the fibroblast growth factor family, which has a unique secretion property. Mol. Cell. Biol. 13, 1993:4251-4259.

168 Mohn KL, Melby AE, Tewari DS, Laz TM, Taub R: The gene encoding rat insulinlike growth factor-binding protein 1 is rapidly and highly induced in regenerating liver. Mol. Cell. Biol. 11, 1991:1393-1401.

169 Momose H, Kakinuma H, Shariff SY, Mitchell GB, Rademaker A, Oyasu R: Tumor-promoting effect of urinary epidermal growth factor in rat urinary bladder carcinogenesis. Cancer Res. 51, 1991:5487-5490.

170 Montesano R, Matsumoto K, Nakamura T, Orci L: Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell 67, 1991:901-908.

171 Moodie SA, Willumsen BM, Weber MJ, Wolfman A: Complexes of Ras.GTP with Raf-1 and Mitogen-Activated Protein kinase kinase. Science 260, 1993:1658-1661.

172 Moore BP, Hocks RM, Knowles MA, Redgrave S: Metabolism and binding of benzo(a)pyrene and 2-acetylaminofluorene by short-term organ cultures of human and rat bladder. Cancer Res. 42, 1982: 642-648.

173 Mori S, Akiyama T, Yamada Y, Morishita Y, Sugawara I, Toyoshima K, Yamamoto T: C-erbB-2 gene product, a membrane protein commonly expressed on human fetal epithelial cells. Lab. invest. 61, 1989:93-97.

174 Moriyama M, Akiyama T, Yamamoto T, Kawamoto T, Kato T, Sato K, Watanuki T, Hikage T, Katsuta N, Mori S: Expression of c-erbB 2 gene product in urinary bladder cancer. J. Urol. 145, 1991:423-427.

175 Moses HL, Yang EY, Pietenpol JA: TGF-ß stimulation and inhibition of cell proliferation: new mechanistic insights. Cell 63, 1990:245-247.

176 Muller WJ, Lee FS, Dickson C, Peters G, Pattengale P, Leder P: The *int*-2 gene product acts as an epithelial growth factor in transgenic mice. EMBO J. 9, 1990:907-913.

177 Mustoe TA, Pierce GF, Morishima C, Deuel TF: Growth factor-induced acceleration of tissue repair through direct and inductive activities in a rabbit dermal ulcer model. J. Clin. Invest. 87, 1991:694-703.

178 Nabel EG, Yang Z, Plautz G, Forough R, Zhan X, Haudenschild CC, Maciag T, Nabel GJ: Recombinant fibroblast growth factor-1 promotes intimal hyperplasia and angiogenesis in arteries *in vivo*. Nature 362, 1993:844-846.

179 Nakamura T, Nawa K, Ishihara A: Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biocehm. Biophys. Res. Commun. 122, 1984:1450-1459.

180 Nakamura T: Structure and function of hepatocyte growth factor. Progr. Growth Factor Res. 3, 1991:67-85.

181 Neal DE, Sharples L, Smith K, Fennelly J, Hall RR, Harris AL: The epidermal growth factor receptor and the prognosis of bladder cancer. Cancer 65, 1990:1619-1625.

182 Neufeld G, Gospodarowicz D: Basic and acidic fibroblast growth factors interact with the same cell surface receptors. J. Biol. Chem. 261, 1986:5631-5637.

183 New BA, Yeoman LC: Identification of basic fibroblast growth factor sensitivity and receptor and ligand expression in human colon tumor cell lines. J. Cell. Physiol. 150, 1992:320-326.

184 Niall M, Ryan GB, O'Brien BMcC: The effect of epidermal growth factor on wound healing in mice. J. Surg. Res. 33, 1982:164-169.

185 Nickoloff BJ, Mitra RS, Riser BL, Dixit VM, Varani J: Modulation of keratinocyte motility. Correlation with production of extracellular matrix molecules in response to growth promoting and antiproliferative factors. Am. J. Pathol. 132, 1988:543-551.

186 Nielsen FC, Wang E, Gammeltoft S: Receptor binding, endocytosis, and mitogenesis of insulin-like growth factors I and II in fetal rat brain neurons. J. Neurochem. 56, 1991:12-21.

187 Nielsen FC: The molecular and cellular biology of insulin-like growth factor II. Prog. Growth Factor Res. 4, 1992:257-290.

188 Niman HL, Thompson AMH, Yu A, Markman M, Willems JJ, Herwig KR, Habib NA, Wood CB, Houghten RA, Lerner RA: Anti-peptide antibodies detect oncogene-related proteins in urine. Proc. Natl. Acad. Sci. USA 82, 1985:7924-7928.

189 Oka Y, Czech MP: The type II insulin-like growth factor receptor is internalized and recycles in the absence of ligand. J. Biol. Chem. 261, 1986:9090-9093.

190 Olivier JP, Raabe T, Henkemeyer M, Dickson B, Mbamalu G, Margolis B, Schlessinger J, Hafen E, Pawson T: A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. Cell 73, 1993:179-191.

191 Östman A, Thyberg J, Westermark B, Heldin C-H: PDGF-AA and PDGF-BB biosynthesis: proprotein processing in the Golgi complex and lysosomal degradation of PDGF-BB retained intracellularly. J. Cell Biol. 118, 1992:509-519.

192 Paterno GD, Gillespie LL, Dixon MS, Slack JMW, Heath JK: Mesoderm-inducing properties of INT-2 and kFGF: two oncogene-encoded growth factors related to FGF. Development 106, 1989:79-83.

193 Piepkorn M, Lo C, Plowman G: Amphiregulin-dependent proliferation of cultured human keratinocytes: autocrine growth, the effects of exogenous recombinant cytokine, and apparent requirement for heparin-like glycosaminoglycans. J. Cell. Physiol. 159, 1994:114-120.

194 Pietenpol AJ, Holt JT, Stein RW, Moses HL: Transforming growth factor ß1 suppression of c-*myc* gene transcription: role in inhibition of keratinocyte proliferation. Proc. Natl. Acad. Sci. USA 87, 1990:3758-3762.

195 Plowman GD, Green JM, McDonald VL, Neubauer MG, Disteche CM, Todaro GJ, Shoyab M: Mol. Cell. Biol. 10, 1990:1969-1981.

196 Plowman GD, Culouscou J-M, Whitney GS, Green JM, Carlton GW, Foy L, Neubauer MG, Shoyab M: Ligand-specific activation of HER4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family. Proc. Natl. Acad. Sci. USA 90, 1993:1746-1750.

197 Poller DN, Spendlove I, Baker C, Church R, Ellis IO, Plowman GD, Mayer RJ: Production and characterization of a polyclonal antibody to the c-erbB-3 protein: examination of c-erbB-3 protein expression in adenocarcinomas. J. Pathol. 168, 1992:275-280.

198 Popliker M, Shatz A, Avivi A, Ullrich A, Schlessinger J, Webb CG: Onset of endogenous synthesis of epidermal growth factor in neonatal mice. Dev. Biol. 119, 1987:38-44.

199 Poupko JM, Radomski JL, Hearn WL: Bovine bladder mucosa microsomal cytochrome P-450 and 4-aminobiphenyl N-hydroxylase activity. Cancer Res. 41, 1981:1306-1310.

200 Prigent SA, Lemoine NR, Hughes CM, Plowman GD, Selden C, Gullick WJ: Expression of the c-erbB-3 protein in normal human adult and fetal tissues. Oncogene 7, 1992:1273-1278.

201 Pusztai L, Lewis CE, Lorenzen J, O'D McGee J: Growth factors: regulation of normal and neoplastic growth. J. Pathol. 169, 1993:191-201.

202 Raghavan D, Shipley WU, Garnick MB, Russell PJ, Richie JP: Biology and management of bladder cancer. N. Engl. J. Med. 322, 1990:1129-1138.

203 Ratliff TL: Role of the immune response in BCG for bladder cancer. Eur. Urol. 21 suppl.

2, 1992:17-21.

204 Ravery V, Jouanneau J, Gil Diez S, Abbou CC, Caruelle JP, Barritault D, Chopin DK: Immunohistochemical detection of acidic fibroblast growth factor in bladder transitional cell carcinoma. Urol. Res. 20, 1992:211-214.

205 Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, Fauci AS: Transforming growth factor type ß: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. Proc. Natl. Acad. Sci. 83, 1986:4167-4171.

206 Roberts CJ, Birkenmeier TM, McQuillan JJ, Akiyama SK, Yamada SS, Chen W-T, Yamada KM, McDonald JA: Transforming growth factor ß stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. J. Biol. Chem. 263, 1988:4586-4592.

207 Roberts AB, Rosa F, Roche, NS, Coligan JE, Garfield M, Rebbert ML, Kondaiah P, Danielpour D, Kehrl JH, Wahl SM, Dawid IB, Sporn MB: Isolation and characterization of TGF-ß2 and TGF-ß5 from medium conditioned by *Xenopus* XTC cells. Growth Factors 2, 1990:135-147.

208 Rollins BJ, Morrison ED, Usher P, Flier JS: Platelet-derived growth factor regulates glucose transporter expression. J. Biol. Chem. 263, 1988:16523-16526.

209 Ross R, Raines EW, Bowen-Pope DF: The biology of platelet-derived growth factor. Cell 46, 1986:155-169.

210 Rotwein P: Structure, evolution, expression and regulation of insulin-like growth factors I and II. Growth Factors 5, 1991:3-18.

211 Rozakis-Adcock M, Fernley R, Wade J, Pawson T, Bowtell D: The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature 363, 1993:83-85.

212 Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA: Purification and characterization of a newly identified growth factor specific for epithelial cells. Proc. Natl. Acad. Sci. USA 86, 1989:802-806.

213 Sacco O, Romberger D, Rizzino A, Beckmann JD, Rennard SI, Spurzem JR: Spontaneous production of transforming growth factor-ß2 by primary cultures of bronchial epithelial cells. J. Clin. Invest. 90:1992:1379-1385.

214 Sanidas EE, Filipe MI, Linehan J, Lemoine NR, Gullick WJ, Rajkumar T, Levison DA: Expression of the c-erbB-3 gene product in gastric cancer. Int. J. Cancer 54, 1993:935-940.

215 Sariban E, Sitaras NM, Antoniades HN, Kufe DW, Pantazis P: Expression of platelet-derived growth factor (PDGF)-related transcripts and synthesis of biologically active PDGF-like proteins by human malignant epithelial cell lines. J. Clin. Invest. 82, 1988:1157-1164.

216 Schultz GS, White M, Mitchell R, Brown G, Lynch J, Twardzik DR, Todaro GJ: Epithelial wound healing enhanced by transforming growth factor-α and vaccinia growth factor. Science

235, 1987:350-352.

217 Schultz GS, Rotatori DS, Clark W: EGF and TGFα in wound healing and repair. J. Cell. Biochem. 45, 1991:346-352.

218 Seppä H, Grotendorst GR, Seppä S, Schiffmann E, Martin GR: The platelet-derived growth factor is chemotactic for fibroblasts. J. Cell Biol. 92, 1982:584-588.

219 Siddle K: The insulin receptor and type I IGF receptor: comparison of structure and function. Prog. Growth Factor Res. 4, 1992:301-320.

220 Sitaras NM, Sariban E, Bravo M, Pantazis P, Antoniades HN: Constitutive production of platelet-derived growth factor-like proteins by human prostate carcinoma cell lines. Cancer Res. 48, 1988:1930-1935.

221 Skolnik EY, Batzer A, Li N, Lee C-H, Lowenstein E, Mohammadi M, Margolis B, Schlessinger J: The function of GRB2 in linking the insulin receptor to Ras signaling pathways. Science 260, 1993:1953-1955.

222 Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin Wj, Stuart SG, Udove J, Ullrich A: Studies of HEr-2/neu proto-oncogene in human breast and ovarian cancer. Science 244, 1989:707-712.

223 Soley M, Hollenberg MD: Epidermal growth factor (urogastrone)-stimulated gluconeogenesis in isolated mouse hepatocytes. Arch. Biochem. Biophys. 255, 1987:136-146.

224 Stark KL, McMahon JA, McMahon AP: FGFR-4, a new member of the fibroblast growth factor receptor family, expressed in the definitive endoderm and skeletal muscle lineages of the mouse. Development 113, 1991:641-651.

225 Sutherland AE, Sanderson RD, Mayes M, Seibert M, Calarco PG, Bernfield M, Damsky CH: Expression of syndecan, a putative low affinity fibroblast growth factor receptor, in the early mouse embryo. Development 113, 1991:339-351.

226 Tajima H, Matsumoto K, Nakamura T: Regulation of cell growth and motility by hepatocyte growth factor and receptor expression in various cell species. Exp. Cell Res. 202, 1992:423-431.

227 Tanaka A, Miyamoto K, Minamino N, Takeda M, Sato B, Matsuo H, Matsumoto K. Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. Proc. Natl. Acad. Sci. (USA) 89, 1992:8928-8932.

228 Taverna D, Groner B, Hynes NE: Epidermal growth factor receptor, platelet-derived growth factor receptor, and c-erbB-2 receptor activation all promote growth but have distinct effects upon mouse mammary epithelial cell differentiation. Cell Growth Differ. 2, 1991:145-154.

229 Terzaghi-Howe M: Changes in response to, and production of, transforming growth factor type ß during neoplastic progression in cultured rat tracheal epithelial cells. Carcinogenesis 10, 1989:973-980.

230 Theillet C, Le Roy X, De Lapeyière O, Grosgeorges J, Adnane J, Raynuad SD, Simony-Lafontaine J, Goldfarb M, Escot C, Birnbaum D, Gaudray P. Amplification of FGF-related genes in human tumors: possible involvement of HST in breast carcinomas. Oncogene 4, 1989:915-922.

231 Theodorescu D, Cornil I, Fernandez BJ, Kerbel RS: Overexpression of normal and mutated forms of *HRAS* induces orthotopic bladder invasion in a human transitional cell carcinoma. Proc. Natl. Acad. Sci. USA 87, 1990:9047-9051.

232 Tsuda L, Inoue YH, Yoo M-A, Mizuno M, Hata M, Lim Y-M, Adachi-Yamada T, Ryo H, Masamune Y, Nishida Y: A protein kinase similar to MAP kinase activator acts downstream of the Raf kinase in Drosophila. Cell 72, 1993:407-414.

233 Tucker GC, Delouvée A, Jouanneau J, Gavrilovic J, Moens G, Vallés AM, Thierry JP: Amplification of invasiveness in organotypic cultures after NBT-II rat bladder carcinoma stimulation with in vitro scattering factors. Invasion and Metastasis 11, 1991:297-309.

234 Uchida K, Samma S, Rinsho K, Warren JR, Oyasu R: Stimulation of epithelial hyperplasia in rat urinary bladder by Escherichia coli cystitis. J. Urol. 142, 1989:1122-1126.

235 Ullrich A, Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. Cell 61, 1990:203-212.

236 Vainikka S, Partanen J, Bellosta P, Coulier F, Basilico C, Jaye M, Alitalo K: Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction. EMBO J. 11, 1992:4273-4280.

237 Valius M, Kaziauskas A: Phospholipase C-γ1 and phosphatidylinositol 3 kinase are the downstream mediators of the PDGF receptor's mitogenic signal. Cell 73, 1993:321-334.

238 Vallés AM, Boyer B, Badet J, Tucker GC, Barritault D, Thierry JP: Acidic fibroblast growth factor is a modulator of epithelial plasticity in a rat bladder carcinoma cell line. Proc. Natl. Acad. Sci. USA 87, 1990:1124-1128.

239 Vanderslice RR, Boyd JA, Eling TE, Philpot RM: The cytochrome P-450 monooxygenase system of rabbit bladder mucosa: enzyme components and isozyme 5-dependent metabolism of 2-aminofluorene. Cancer Res. 45, 1985:5851-5858.

240 Vaughan TJ, Pascall JC, Brown KD: Tissue distribution of mRNA for heparin-binding epidermal growth factor. Biochem. J. 287, 1992:681-684.

241 Versnel MA, Hagemeijer A, Bouts MJ, Van der Kwast ThH, Hoogsteden HC: Expression of c-*sis* (PDGF B-chain) and PDGF A-chain genes in ten human malignant mesothelioma cell lines derived from primary and metastatic tumors. Oncogene 2, 1988:601-605.

242 Versnel MA, Claesson-Welsh L, Hammacher A, Bouts MJ, Van der Kwast TH, Eriksson A, Willemsen R, Weima SM, Hoogsteden HC, Hagemeijer A, Heldin C-H: Human malignant mesothelioma cell lines express PDGF beta-receptors whereas cultured normal mesothelial cells express predominantly PDGF alpha-receptors. Oncogene 6, 1991:2005-2011.

243 Vojtek AB, Hollenberg SM, Cooper JA: Mammalian Ras interacts directly with the

serine/threonine kinase Raf. Cell 74, 1993:205-214.

244 Wang XF, Lin HY, Ng-Eaton E, Downward J, Lodish HF, Weinberg RA: Expression cloning and characterisation of TGFß type III receptor. Cell 67, 1991:797-805.

245 Warne PH, Viciana PR, Downward J: Direct interaction of Ras and the amino-termianl region of Raf-1 *in vitro*. Nature 364, 1993:352-355.

246 Waterfield MD, Scrace T, Whittle N, Stroobant P, Johnsson A, Wasteson Å, Westermark B, Heldin C-H, Huang JS, Deuel TF: Platelet-derived growth factor is structurally related to the putative transforming growth factor $p28^{sis}$ of simian sarcoma virus. Nature 304, 1983:35-39.

247 Werner S, Duan D-SR, De Vries C, Peters KG, Johnson DE, Williams LT: Differential splicing in the extracellular region of fibroblast growth factor receptor 1 generates receptor variants with different ligand-binding specifities. Mol. Cell. Biol. 12, 1991:82-88.

248 Wilcox JN, Derynck R: Developmental expression of transforming growth factors alpha and beta in mouse fetus. Mol. Cell. Biol. 8, 1988:3415-3422.

249 Wilke MS, Hsu BM, Wille JJ, Pittelkow MR, Scott RE: Biologic mechanisms for the regulation of normal keratinocyte proliferation and differentiation. Am. J. Pathol. 131, 1988:171-181.

250 Wilkinson DG, Peters G, Dickson C, McMahon AP: Expression of the FGF-related proto-oncogene *int*-2 during gastrulation and neurulation in the mouse. EMBO J. 7, 1988:691-695.

251 Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J: Mechanism of activation of the TGF-ß receptor. Nature 370, 1994:341-347.

252 Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM: Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64, 1991:841-848.

253 Yoshida T, Miyagawa K, Odagiri H, Sakamoto H, Little PFR, Terada M, Sugimura T: Genomic sequence of *hst*, a transforming gene encoding a protein homologous to fibroblast growth factors and the *int*-2-encoded protein. Proc. Natl. Acad. Sci. USA 84, 1987:7305-7309.

254 Yura Y, Hayashi O, Kelly M, Oyasu R: Identification of epidermal growth factor as a component of the rat urinary bladder tumor-enhancing urinary fractions. Cancer Res. 49, 1989:1548-1553.

255 Zhan X, Bates B, Hu X, Goldfarb M: The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. Mol. Cell. Biol. 8, 1988:3487-3495.

256 Zhang X-F, Settleman J, Kyriakis JM, Takeuchi-Suzuki E, Elledge SJ, Marshall MS, Bruder JT, Rapp UR, Avruch J: Normal and oncogenic p21*ras* proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 364, 1993:308-313.

Chapter 2.

MULTIPARAMETER ANALYSIS OF PRIMARY EPITHELIAL CULTURES GROWN ON CYCLOPORE MEMBRANES.

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Technical Note

Multiparameter Analysis of Primary Epithelial Cultures Grown on Cyclopore Membranes[1]

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The use of porous membranes as culture support for epithelial cells has previously been shown to cause functional differentiation of these cells mimicking an in vivo condition, in contrast to culture on plastic. The different materials of which the membranes are made also have different properties, such as transparency, rigidity, and retention of molecules. Cyclopore membranes (polyethylene terephtalate) are permeable, transparent, rigid, and have low protein retention. In this study we examined the applicability of assessing multiple parameters on a single culture of primary epithelial cells on a Cyclopore membrane. Cultures of transitional epithelial cells on these membranes differentiate into an organoid-like epithelium. We were able to perform morphometric analysis during and after cell culture and to quantitate proliferation and differentiation by double immunoenzymatic staining. On these cultures, quantitative radiochemical analysis could also be achieved, retaining the morphology and the immunohistochemical staining. Cross-sections of paraffin-embedded and plastic-embedded cultures were analyzed qualitatively by light and transmission electron microscopy, respectively. Finally, cytokeratins in these cultures could also be visualized by immunofluorescence analysis. This suitability for simultaneous assessment of both qualitative and quantitative parameters on a single cell culture grown on a Cyclopore membrane reduces the need of biological materials and may lead to better insight into physiological processes. (*J Histochem Cytochem 42:277–282, 1994*)

KEY WORDS: Immunohistochemistry; Morphology; Morphometry; Mouse; Primary culture; Epithelium; Porous membrane.

Introduction

Several kinds of culture supports are available for in vitro study of proliferation and differentiation of epithelial and mesenchymal cells. To mimic in vivo conditions as closely as possible, culture substrates have been developed that are permeable to medium components. In contrast to cell cultures on conventional non-porous plastic, polarization and multilayering of cells grown on (extracellular matrix-coated) permeable membranes are observed, as these membranes allow diffusion of nutrients to both the basolateral and the apical side of the cells.

Collagen gels have often been used to improve in vitro culture conditions (13). Disadvantages of the use of collagen gels as permeable culture substrates are their more complicated handling and their variation in molecular composition or structure. In addition to collagen gels, porous membranes made from a variety of materials have become increasingly available as culture supports to study morphology (4,9), transport of (pharmacological) agents across cell layers (7,11), co-cultivation of different cell types (14,16), and cell invasiveness (12).

Porous membranes are composed of different materials such as cellulose ester, nylon, polycarbonate, and polyethylene terephtalate. The composition of these membranes, the pore density, and the pore size influence their properties with respect to transparency, rigidity, and retention of proteins. Their suitability for light and electron microscopy and for immunohistochemistry may vary accordingly. Porous polyethylene terephtalate membranes are transparent, permeable, and rigid. These membranes have been successful in supporting the functional differentiation of CaCo2 colon carcinoma cells (5).

This study was designed to evaluate whether multiple quantitative and qualitative (morphological) parameters can be assessed simultaneously on organotypic cultures grown on polyethylene terephtalate (Cyclopore) membranes. The tested parameters include light (LM) and transmission electron microscopy (TEM) on cross-sections, immunofluorescence, double-staining immunohistochemistry for proliferation and differentiation, morphometry, and liquid scintillation counting of incorporated tritiated thymidine.

Materials and Methods

Chemicals. Culture media and supplements were purchased from Flow Laboratories (Zwanenburg, Netherlands) and Sigma (St Louis, MO), respec-

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tively. Tritiated thymidine was purchased from Amersham ('sHertogenbosch, Netherlands). Scintillation toluene, PicoFluor, and Ultima Gold were obtained from Packard (Groningen, Netherlands). Chain-specific antibody against cytokeratin 18 (RGE 53) was purchased from Organon Technica (Oss, Netherlands). Chain-specific antibody against cytokeratin 19 (LP2K) and anti-BrdU antibody were kindly donated by Prof. Dr. F. C. S. Ramaekers (University of Maastricht, Maastricht, Netherlands).

Cells and Culture. Urinary bladders were dissected from 6–8-week-old female C3H/Law mice and cut into halves. After washing the explants and wiping off excessive medium, the mucosal cell layer was stripped from the muscle layer and submucosa. The mucosal layer was spread on a Cyclopore membrane culture support (Falcon; Becton Dickinson, Mountain View, CA) with the basement membrane facing the support. Culture medium was added and refreshed on alternate days. The primary urothelial cell culture resulted from the cells spreading out of the mucosa. Standard medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F10, supplemented with 10% heat-inactivated fetal calf serum (FCS), 5 µg/ml insulin and transferrin, 50 nM hydrocortisone, 5 ng/ml selenite, 10 µM HEPES, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. Since the pore size and density may affect the cell number (5), we tested the growth of primary epithelial cultures on three different types of 2.5-cm-diameter Cyclopore membranes: membranes with pore size of 0.45 µm and pore density of 1.6×10^6 pores/cm^2 or 1.0×10^8 pores/cm^2, and membranes with pore size of 1 µm and 1.6×10^6 pores/cm^2. Of these, the high pore-density membranes were the least transparent. Optimal adhesion and subsequent outgrowth of epithelial cells were observed only for the membranes with pore size of 0.45 µm and 1.6×10^6 pores/cm^2. Urothelial explants frequently detached from the other types of Cyclopore membranes. Therefore, we used the latter type of membrane for this study.

[^{3}H]-Thymidine and BrdU Incorporation. On termination of the culture, cells were first incubated with 40 µg/ml 5-bromo-2'-deoxyuridine (BrdU) in standard medium for 2 hr. Then this medium was replaced by standard medium containing 20 µCi [^{3}H]-thymidine/ml without Ham's F10 and FCS, in which the cells were cultured for another 2 hr. Next, cells were rinsed with PBS, pH 7.2, with an excess of non-labeled thymidine. The incorporated radioactivity was counted in scintillation liquid using an α,β-scintillation analyzer (Packard 2500 TR; Groningen, Netherlands), luminescence and color-quenching corrected, and expressed as counts/min (cpm) and disintegrations/min (dpm).

Immunohistochemistry and Immunofluorescence. Cultures were rinsed with PBS, pH 7.2, and fixed with ethanol 96% for at least 1 hr. Before the primary anti-BrdU antibody incubation, cultures were treated with HCl and Borax buffer as described (15). Chain-specific cytokeratin and BrdU expression were demonstrated with appropriate dilutions of the primary mouse monoclonal antibodies (MAb) in a conjugated immunoenzyme assay. Secondary rabbit anti-mouse MAb (DAKO; Etten Leur, Netherlands) were either peroxidase-conjugated or alkaline phosphatase-conjugated. Chromogens were 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Fluka; Oud Beijerland, Netherlands) and the diazonium salt Fast red violet LB with naphthol AS-MX phosphate as coupling agent (Sigma), respectively. Azo dye visualization was done according to Burstone (1). All incubations were done while membranes were floating upside down in the incubation mixture. Membranes were mounted in Euparal (ethanol-based) (Chroma; Stuttgart, FRG) on glass slides. Mounting in xylene-based Malinol solubilized the azo dyes, resulting in decolorizing of the red-stained culture. In fluorescence assays either fluorescein isothiocyanate (FITC)-conjugated or tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-mouse secondary MAb (DAKO) were used to examine cytokeratin expression. These membranes were embedded in PBS with glycerin and visualized with a conventional fluorescence microscope. Ethanol-fixed frozen sections of cytokeratin-positive mouse and human bladder served as control for cytokeratin–primary antibody binding and as control for specific antibody binding.

Transmission Electron Microscopy. Confluent cultures were rinsed with PBS, pH 7.2, and fixed with 1.5% w/v glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After rinsing with cacodylate buffer, pH 7.4, cells were post-fixed with 1% w/v OsO_4 and 1.5% w/v potassium ferrocyanide in cacodylate buffer. After dehydration with ethanol, cells were embedded in Epon (Zeiss; Weesp, Netherlands). Ultra-thin sections (0.02 µm) were contrasted on 200-mesh copper grids with uranyl acetate and lead nitrate. Sections were studied with a TEM (Zeiss EM902) at 80 kV.

Morphometry and Image Analysis. The edge of the outgrowth of the explant culture, as visualized by LM, was drawn by hand. The area of outgrowth drawn was measured by image analysis. Quantification of cytokeratin-stained parts of the membrane or drawn outgrowth areas was also done with an image analyzer (IBAS 2000 Zeiss Kontron; Oberkochen, Germany). Cultures and drawn areas, respectively, were visualized with a Hitachi CCTV camera and images were analyzed with the supplied Kontron IBAS1 version 4.4 software program. Cytokeratin-stained areas and outgrowth areas were calculated in mm^2 and expressed as percentage of the maximal outgrowth area.

Results

The transparency of the Cyclopore membranes permitted daily measurement of the expansion of the primary urothelial outgrowth from

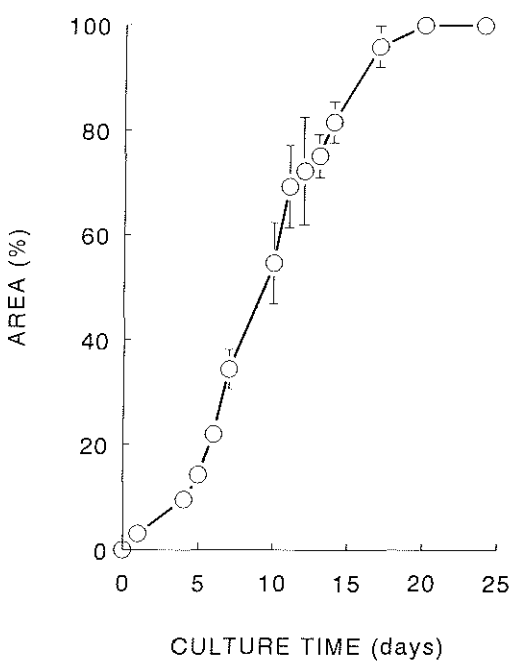


Figure 1. Morphometry of the outgrowth of primary urothelial cultures during cultivation. The outgrowth area was related to the maximal outgrowth area, which was set at 100%, and plotted against the time of culture (in days). Bars = SD (n = 6).

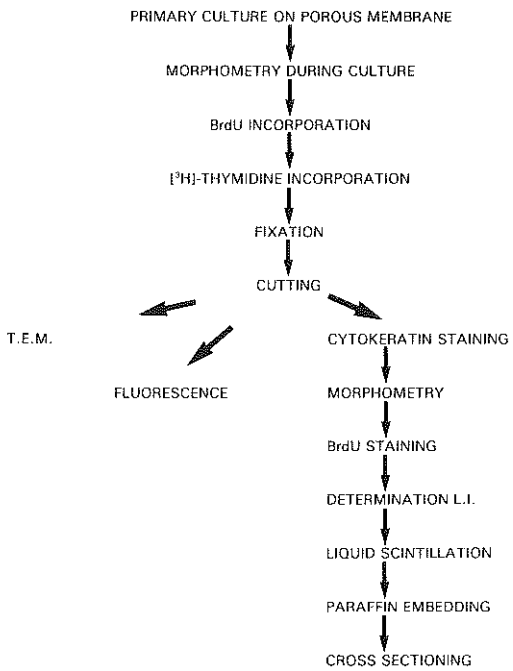


Figure 2. Flow chart outlining the subsequent steps for assessment of the described parameters. T.E.M., transmission electron microscopy; L.I., labeling index.

the inoculated mouse bladder mucosa explants (Figure 1), as the advancing edges of the explant cultures were easy to distinguish. The sequence of procedures performed on the Cyclopore membranes covered by the confluently grown cultures are depicted in a flow chart (Figure 2).

The rigidity of the membranes enabled them to be cut into two or more parts. Fixation in ethanol or acetone for immunohistochemistry or in glutaraldehyde for TEM did not affect the transparency of the membranes. Double immunoenzymatic staining was performed to simultaneously demonstrate nuclear reactivity for BrdU and cytoplasmic staining for cytokeratin 18 or 19. First, cytokeratin expression was visualized with the alkaline phosphatase-coupled secondary antibody, since the best contrast for image analysis was achieved with the chromogen Fast red violet. After mounting of the immunostained membranes on glass slides, LM examination was possible. At high-power magnification the large, superficially located cytokeratin 18-positive cells covered several non-stained underlying cells (Figure 3A). In contrast, the underlying smaller cells were mainly cytokeratin 19 positive (Figure 3B). This indicates that equal permeation of antibodies and reagents to all cell layers of the culture was possible. No adhesion of the immunohistochemical reaction products to the porous membranes occurred. The pores in the supporting membranes were, however, clearly visible due to the diffraction of light. As cytokeratin 18 was expressed in differentiated superficial cells, this expression could be used as a parameter for terminal differentiation of the culture. By image analysis, the relative cytokeratin 18-positive areas were quantitated, showing that about 42.7% of the superficial cells expressed cytokeratin 18 (Table 1). After image analysis BrdU staining was performed with a peroxidase-coupled secondary antibody and DAB as chromogen. Proliferative activity could be localized and quantitated by visualizing and counting the BrdU-stained nuclei and by measuring the uptake of [^{3}H]-thymidine (Table 1).

Table 1. *Multiparameter analysis on urothelial cultures*[a]

Nuclei per 0.15 mm^2	LI (%)	[^{3}H]-Thy (dpm)	CK 18 area (%)
264.3 ± 73.4	1.8 ± 1.5	11,061 ± 1747	42.7 ± 19.1

[a] Quantitation of parameters for proliferation and differentiation on single urothelial cultures. Nuclei are counted in six pre-fixed areas of 0.15 mm^2 on the membrane. The BrdU incorporation [calculated as the labeling index (LI)] and the tritiated thymidine ([^{3}H]-Thy) uptake were taken as parameters of proliferation. The LI is given as the number of BrdU-positive nuclei relative to the total number of nuclei. Incorporation of [^{3}H]-Thy was measured in Ultima Gold and expressed in dpm. Terminal differentiation of the cultures is reflected by the expression of cytokeratin 18. The cytokeratin 18-positive areas (CK 18 area) were calculated by image analysis as percentage of the maximal outgrowth area, which was set at 100%. Bars = SD of five cultures.

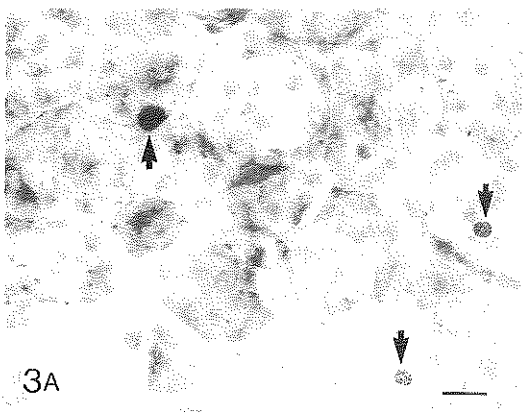
Alternatively, when cultures primed with primary antibodies against cytokeratin 18 or 19 were incubated with FITC- or TRITC-conjugated secondary antibodies, a clear cytokeratin expression pattern was observed (Figure 4) similar to the pattern shown in Figure 3. Compared with the specific signal, the background fluorescence derived from the membrane or pores in the membrane was low for both types of fluorescent dyes, even at high magnification (× 1000).

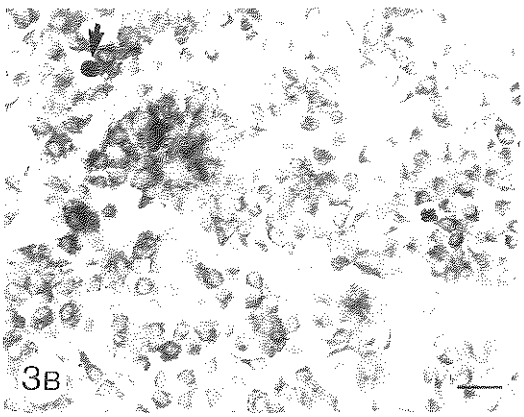
To visualize the immunostaining on cross-sections after liquid scintillation counting of the membranes, it was necessary that the precipitated chromogens would not dissolve by immersion in the scintillation liquid. Several brands of scintillation liquids were tested. Scintillation toluene and, to a lesser extent, PicoFluor did destain the cultures, but Ultima Gold did not. No differences in counting efficiency were observed for the culture tested: the number of cpm and the concurrent dpm were similar within a given culture regardless of the type of scintillation liquid and quantity of incorporated radioactivity (Table 2). Furthermore, background levels of blank

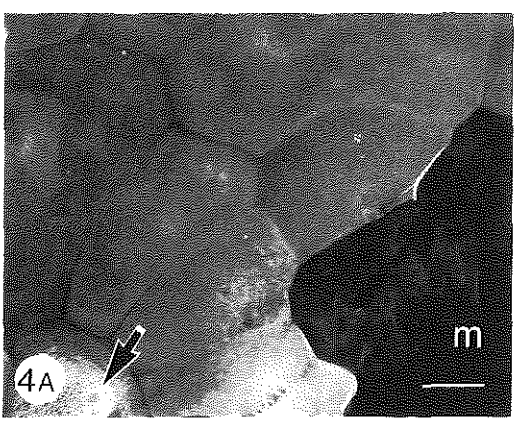
Table 2. *Comparison of scintillation liquids in [^{3}H]-thymidine measurement*

	Culture				No cells	
Liquid	1	2	3	4	Exposed	Not exposed
Toluene	7945	7254	5622	626	8	0
DPM	23,317	21,492	18,005	1960	23	0
PicoFluor	6719	6416	4819	563	8	0
DPM	23,203	22,228	16,544	1880	26	0
Ultima Gold	5787	6373	4396	530	3	0
DPM	22,068	20,244	15,211	1780	11	0

[a] Use of different scintillation liquids for counting incorporated [^{3}H]-Thy into four urothelial primary cultures, one membrane without cells but exposed to [^{3}H]-Thy, and one membrane without cells and not exposed to [^{3}H]-Thy. Culture 4 was treated differently in receiving 4 ng/ml transforming growth factor-β for 5 days. Incorporations are given in cpm and in disintegrations per minute (DPM).







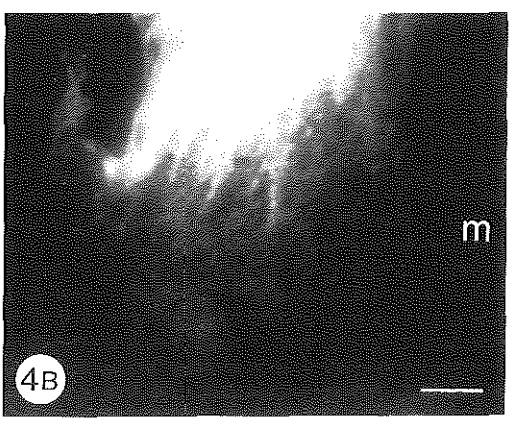


Figure 3. The upper surface of (**A**) a cytokeratin 18-stained and (**B**) a cytokeratin 19-stained urothelial culture, counterstained with hematoxylin. Large superficial cells stain for cytokeratin 18 and reside on underlying, cytokeratin 19-stained smaller cells. BrdU-stained nuclei are denoted by a thick arrow. Original magnification × 200. Bars = 50 µm.

Figure 4. Immunofluorescence of (**A**) cytokeratin 18-expressing cells with FITC-conjugated antibody and (**B**) a cytokeratin 19-expressing cell bordering a blank part of the membrane with TRITC-conjugated antibody. Arrow indicates intracellular vacuoles. M, blank membrane. Original magnifications: **A** × 250; **B** × 1000. Bars: **A** = 1.56 µm; **B** = 0.39 µm.

membranes exposed to [^{3}H]-thymidine and of non-exposed blank membranes were low, indicating low retention of thymidine by these membranes.

After measurement of radioactivity, the membranes were embedded in paraffin and cross-sections of the immunostained cultures were made perpendicular to the membrane. The rigidity of the membranes was sufficient for the cutting, and no shrinking of the membrane, causing disruption of the cultures, was observed. In cross-sections the BrdU-stained nuclei were found in the basal cell layer, while cytokeratin 18-stained cells covered the cell culture. The best results for photography were obtained when BrdU staining (with Fast red violet as chromogen) was performed before cytokeratin staining (with DAB as chromogen) (Figure 5). This sequence resulted in more intense BrdU staining. A slight solubilization of the azo dye precipitate, but not the polymerized DAB, occurred during the process of removing the paraffin with petroleum benzine.

TEM on the epithelial cells covering the membrane could be performed without the need to separate the culture from the porous membrane. In ultra-thin cross-sections the multilayered cell culture and preserved cell organelles could be visualized. The membrane detached from the cell layers during ultra-thin sectioning. Figure 6A shows a culture consisting of a layer of smaller basal cells, the cuboid intermediate cell layer, and the thin superficial cell layer.

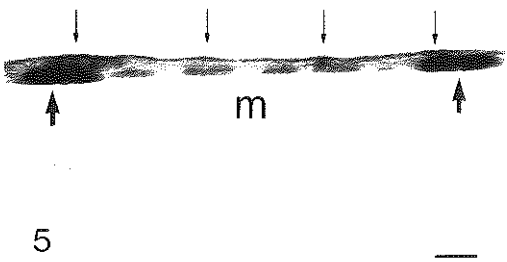


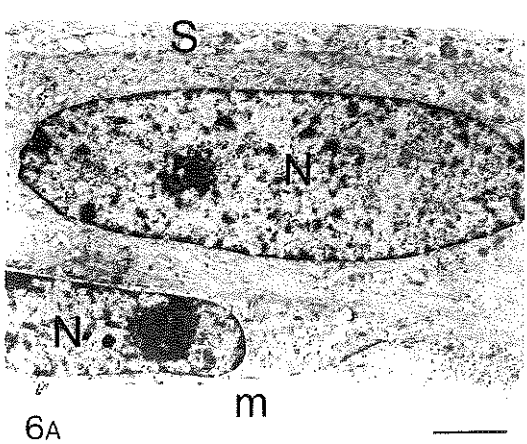
Figure 5. Cross-section of a urothelial cell cultured on a Cyclopore membrane. BrdU stained basal cells (thick arrows) and cytokeratin 18 only superficial cells in arrows). M, membrane. Original magnification x 1000. Bar = 10 µm.

Figure 6B shows subcellular details such as organelles and intermediate filaments.

Discussion

The results of this study demonstrate that a variety of parameters can be assessed on a single epithelial culture grown on a porous membrane. The epithelial primary culture shows features of the murine bladder urothelium in vivo: three cell layers, the flattened, cytokeratin 18-positive, upper cell layer representing the superficial urothelial cells; the cuboid intermediate cell layer; and the smaller basal cells in the basal cell layer (6). This multilayering and differentiation of epithelial cells has also been observed with other porous membranes (16), but not with plastic dishes (4). Therefore, the permeability of the membrane enables medium components also to reach the basolateral side of the cells, resulting in an optimal culture system, in contrast to plastic dishes. The transparency of the Cyclopore membranes is an advantage over cellulose acetate or polycarbonate membranes when morphometric analysis is to be performed both during and after cultivation. Furthermore, the 0.45 µm-pore size Cyclopore membranes are more rigid than Transwell-COL membranes. As a consequence, the Cyclopore membranes do not crinkle when cut from the insert ring or during embedding in paraffin or plastic, in contrast to Transwell-COL membranes (2). Therefore, one membrane can be easily divided into more pieces for different fixations without damage to the culture. If cultures are fixed with acetone, the Transwell-COL membranes but not the Cyclopore membranes also have the disadvantage that the membranes separate from the insert ring resulting in crinkling of the membrane. A disadvantage of the Cyclopore membrane is its impermeability to Epon, resulting in detachment of the membrane during ultra-thin sectioning for TEM, although this artifact has no consequence for visualization of the culture by TEM.

From previous studies it is apparent that immunohistochemistry cannot be performed on all types of porous membranes. With nitrocellulose membranes, Butor et al. (3) showed that immunological reagents have less access to the basal side of monolayer cultures of renal MDCK epithelial cells. Immunoassays performed on nitrocellulose membranes yielded poor results because of high back-



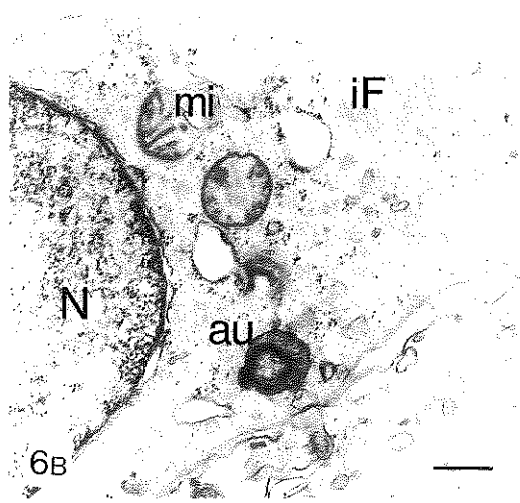


Figure 6. TEM showing (A) a cross-section of a urothelial culture with three different cell layers and (B) subcellular detail. N, nucleus; S, superficial cell; m, original localization of the membrane; mi, mitochondrion; iF, intermediate filaments; au, autophagic body. Original magnification: A x 3000; B x 20,000. Bars: A = 2.5 µm; B = 0.4 µm.

ground (10). The present study demonstrates that immunohistochemistry can be performed on Cyclopore membranes with low retention of immunohistochemical compounds. Although cultures were multilayered, good access to basal cell layers was achieved, as visualized for BrdU staining in the basal cells. Supporting evidence for good access to all cell layers was also provided by staining of cytokeratin 19 in basal and intermediate cell layers (not shown).

When antibodies conjugated to fluorescent dyes are used with polycarbonate membranes, light is diffracted by the pores in the membrane, necessitating the use of a confocal laser scanning microscope (3). Since Cyclopore membranes also contain pores that diffract light in a conventional LM, we expected similar problems for these membranes, although our results showed that FITC- or TRITC-labeled antibodies bound specifically to anti-cytokeratin antibodies. A clear distinction between fluorescence in superficial and basal cells could be made. Furthermore, the fluorescence background of the membrane was low and the pores in the membrane barely quenched the fluorescence signal. This demonstrates that Cyclopore membranes are also useful in fluorescence assays.

Measurement of the cellular incorporation of isotopes has been performed previously on cells cultured on Transwell-COL membranes; these cells were scraped off of the membranes (8,17). In this study we showed that liquid scintillation counting can be performed on cultures that are still attached to the membrane. Prerequisites for effective scintillation analysis on membrane-attached cells are the resistance of the membrane to the scintillation liquid and the transparency of the membrane in the scintillation liquid, because non-transparent membranes will undoubtedly quench the scintillation measurement. The immunohistochemical azo dye stainings are solubilized in toluene scintillation liquid or PicoFluor, but this could be prevented by using Ultima Gold as scintillation liquid. In contrast, the DAB stainings are not affected by either scintillation liquid (not shown). In conclusion, the use of porous membranes, in particular Cyclopore membranes, offers the opportunity to obtain a single differentiated, organoid-like cell culture on which several different parameters can be assessed. These parameters can either be quantified (e.g., morphometry, counting of nuclei or incorporation of radiochemicals) or used to assess qualitative aspects of the culture (e.g., immunofluorescence, cross-sectioning, and TEM). In turn, this multiparameter analysis on one cell or cell culture may help to provide better insight into physiological (inter)cellular processes. The analysis of several parameters on one single cell culture reduces the need for biological material.

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Literature Cited

1. Burstone MS: Enzyme histochemistry and its application in the study of neoplasms. New York, London, Academic Press, 1962
2. Butor C, Davoust J: Apical to basolateral surface area ratio and polarity of MDCK cells grown on different supports. Exp Cell Res 203:115, 1992
3. Butor C, Stelzer EHK, Sonnenberg A, Davoust J: Apical and basal Forssman antigen in MDCK II cells: a morphological and quantitative study. Eur J Cell Biol 56:269, 1991
4. Chlapowski FJ, Haynes L: The growth and differentiation of transitional epithelium in vitro. J Cell Biol 83:605, 1979
5. Halleux C, Schneider Y-J: Iron absorption by intestinal epithelial cells: 1. CaCo2 cells cultivated in serum-free medium, on polyethyleneterephtalate microporous membranes, as an in vitro model. In Vitro Cell Dev Biol 27A:293, 1991
6. Hicks RM: The mammalian urinary bladder: an accommodating organ. Biol Rev 50:215, 1975
7. Hidalgo IJ, Raub TJ, Borchardt RT: Characterization of the human colon carcinoma cell line (CaCo2) as a model system for intestinal epithelial permeability. Gastroenterology 96:736, 1989
8. Jalal F, Lemay G, Zollinger M, Berteloot A, Boileau G, Crine P: Neutral endopeptidase, a major brush border protein of the kidney proximal nephron, is directly targeted to the apical domain when expressed in Madin–Darby canine kidney cells. J Biol Chem 266:19826, 1991
9. Knowles MA, Finesilver A, Harvey AE, Berry RJ, Hicks RM: Long-term organ culture of normal human bladder. Cancer Res 43:374, 1983
10. Mostov KE, De Bruyn Kops A, Deitcher DL: Deletion of the cytoplasmic domain of the polymeric immunoglobulin receptor prevents basolateral localization and endocytosis. Cell 47:359, 1986
11. Narula P, Xu M, Kuang K, Akiyama R, Fischbarg J: Fluid transport across cultured bovine corneal endothelial cell monolayers. Am J Physiol 262:C98, 1992
12. Repesh LA: A new in vitro assay for quantitating tumor cell invasion. Invasion Metastasis 9:192, 1989
13. Reznikoff CA, Loretz LJ, Pesciotta DM, Oberley TD, Ignjatovic MM: Growth kinetics and differentiation in vitro of normal human uroepithelial cells on collagen gel substrates in defined medium. J Cell Physiol 131:285, 1987
14. Robinson SP, Jordan VC: The paracrine stimulation of MCF-7 cells by MDA-MB-231 cells: possible role in antiestrogen failure. Eur J Cancer Clin Oncol 25:493, 1989
15. Schutte B, Reynders MMJ, Van Assche CLMVJ, Hupperets PSGJ, Bosman FT, Blijham GH: Studies with anti-bromodeoxyuridine antibodies. Cytometry 8:372, 1987
16. Valyi-Nagi IT, Murphy GF, Mancianti M-L, Whitaker D, Herlyn M: Phenotypes and interactions of human melanocytes and keratinocytes in an epidermal reconstruction model. Lab Invest 62:314, 1990
17. Van 't Hof W, Silvius J, Wieland F, van Meer G: Epithelial sphingolipid sorting allows for extensive variation of the fatty acid chain and the sphingosine backbone. Biochem J 283:913, 1992

Chapter 3.

CHARACTERIZATION OF DISTINCT FUNCTIONS FOR GROWTH FACTORS IN MURINE TRANSITIONAL EPITHELIAL CELLS IN PRIMARY ORGANOTYPIC CULTURE.

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ABSTRACT

Although previous studies indicate that growth factors can affect several physiological processes in epithelia, their role in the biological dynamics of transitional epithelium of the bladder is not yet established. This study investigates the functional consequences of a direct action of EGF, TGFß, FGF-1, FGF-2, PDGF-AA, and PDGF-BB on mouse urothelium in organoid-like primary cultures. Confluent and non-confluent cultures served as a model for intact and regenerating urothelium, respectively. EGF and FGF-1 stimulated in both models under serum-free conditions the BrdU and [^{3}H]-thymidine incorporation. This resulted in an increase in the number of cell layers, but the cultures assumed a less organoid-like morphology. In addition, EGF and FGF-1 stimulated the expansion of non-confluent cultures. TGFß inhibited proliferation, caused a decrease in the number of cell layers and blocked expansion. Moreover, TGFß induced the terminal differentiation and apoptosis of urothelial cells. In non-confluent cultures PDGF-BB slightly stimulated the increase in the outgrowth area, but no other effect on the parameters for proliferation and differentiation was observed. FGF-2 and PDGF-AA did not affect any of the studied parameters. These data are consistent with the hypothesis that EGF and FGF-1 can promote wound healing and/or hyperplasia through direct action on the epithelial cells, while TGFß promotes the development of a normal, differentiated transitional epithelium.

INTRODUCTION

Several studies demonstrated that growth factors can be involved in both physiological and pathological processes like embryogenesis [1], regeneration [2,3], and tumorigenesis [3,4]. Growth factors can be synthesized and secreted by a variety of tissues in vivo including urogenital epithelia. Transforming growth factor ß (TGFß) and insulin-like growth factors (IGF) are produced in kidney epithelium [5,6], while epidermal growth factor (EGF) has been found in high concentrations in the urine [7]. The presence of transforming growth factor α (TGFα) [8] and fibroblast growth factor (FGF) [9] in the urine was found in association with bladder and kidney cancer. Little is known about the occurrence of platelet-derived growth factor (PDGF) in normal epithelia including those of the urogenital tract. Recent studies of Antoniades et al [2] and Floege et al [10] demonstrated PDGF-B and/or PDGF type ß receptor expression in skin

and renal epithelial cells in vivo only under specific conditions. TGFß has also been found in urine [8] and in kidney and urothelial cells [5,11,12]. Both the presence in urine and the differentiation induction in other epithelial cell types [13,14] suggest that TGFß is analogously implicated in the induction of differentiation of the transitional epithelium. These data provide circumstantial evidence that the distinct growth factors can modulate proliferation and differentiation through direct action on transitional epithelia.

The aim of the present study is to investigate and characterize the direct actions of different growth factors on bladder urothelium, without the interference of in vivo-associated reactions to exogenously added growth factors. Therefore, we employed an organoid-like mouse transitional epithelium cultured on a porous culture substrate, which closely mimicks the mouse urothelium in vivo [15]. This culture model permitted us to study the direct effects of different growth factors on proliferation, differentiation, migration, and apoptosis of mouse transitional epithelial cells. All these parameters could be measured simultaneously on a single culture [15]. Previous studies showed that EGF, TGFß, FGF-1, FGF-2, and PDGF-AA modulated the proliferation of non-tumorigenic murine urothelial cell lines [16], while little is known about their effects on migration or differentiation of transitional epithelial cells [17,18]. These growth factors were therefore included in the present study. Since a different growth factor-mediated regulatory mechanism may operate in regeneration of epithelium [2], we studied two different culture models. In the first in vitro model resembling intact bladder mucosa, the effects of growth factors on confluent cultures were investigated. In the second model, non-confluent, growing cultures were examined as they can be considered to reflect regenerating epithelium [19]. We demonstrated that 1) most of the studied growth factors acted similarly in both models with respect to the examined parameters, 2) EGF and FGF-1 induce proliferation but not differentiation, 3) PDGF may affect migration, but not proliferation or differentiation, and 4) TGFß induces terminal differentiation and apoptosis of urothelium.

MATERIALS AND METHODS

Chemicals

Cell culture media were obtained from Flow Laboratories (Zwanenburg, NL), and media supplements from Sigma (St.Louis, USA). Porcine insulin, mouse EGF, and human $TGF\beta_1$ were purchased from Sigma (St.Louis, USA). Human

FGF-1, PDGF-AA, and PDGF-BB were purchased from Boehringer Mannheim (Almere, NL). Human FGF-2 was obtained from Oncogene Science (Uniondale, USA) and porcine heparin from UBI (Lake Placid, USA). Chain-specific antibody RGE 53 against cytokeratin 18 was obtained from Organon Teknika (Oss, NL), and antibody IIB5 against 5-bromo-2'-deoxyuridine (BrdU) was kindly donated by Prof.Dr. F.C.S. Ramaekers (University of Limburg, Maastricht, NL). Tritiated thymidine was obtained from Amersham ('sHertogenbosch, NL) and Ultima Gold from Packard (Groningen, NL).

Primary cell culture.

Primary cultures of female C3H/Law mouse urothelia were obtained as described previously [15]. In short, mouse urothelium was dissected from the urine bladder and placed onto a 25 mm 0.45 μm pore Cyclopore membrane culture support (Falcon culture insert, Becton-Dickinson, Etten Leur, NL) with the submucosa facing the support. Placed in a 6-well dish, the compartments were filled with routine culture medium [15] consisting of 1:1 Ham's F10 and Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% heat-inactivated foetal calf serum (FCS), 5 μg/ml insulin and transferrin, 50 nM hydrocortisone, 5 ng/ml selenite, 10 μM HEPES, and 100 IU/ml penicillin and 100 μg/ml streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cultures were then grown to 55 to 65 percent of the maximal outgrowth area, or to confluency.

Serum-free medium experiments.

Experiments with growth factors were performed in serum-free medium as decribed previously [16]. This medium is similar to the routine medium without FCS, but supplemented with 0.1% bovine serum albumin (BSA), 4 μM spermine, 4 μM spermidin, 0.1 mM ethanolamine, and 1 μM putrescin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 4 days (confluent cultures) or the indicated culture time (non-confluent cultures). Without insulin, the primary cultures failed to grow and died (data not shown).

In experiments on terminal differentiation and apoptosis, confluent cultures were first incubated during 4 days with 20 ng/ml EGF in serum-free medium and pulse-chased during the final 8 hours with BrdU (see below). Subsequently, cells were incubated during 6 days with 0.5 ng/ml TGFß$_1$ or serum-free medium alone.

In all experiments with growth factors the culture medium was replaced

daily. The human growth factors were shown to modulate the proliferation of mouse urothelial cell lines [16]. We chose to use 20 ng/ml EGF, since this concentration of EGF has also been found in urine of mice [20]. Based upon previous experiments with murine urothelial cell lines [16,17], we chose to use 20 ng/ml of both FGFs and PDGFs. FGF-1 was used in combination with 2 U/ml heparin.

[^{3}H]-thymidine and BrdU Incorporation.

The pulse labelling was done as described previously [15]. In short, upon termination of the culture, cells were firstly incubated with 40 μg/ml BrdU in routine medium for two hours, and subsequently in fresh standard medium containing 20 μCi [^{3}H]-thymidine/ml without Ham's F10 and FCS for another two hours. After rinsing the cells with non-labelled thymidine in phosphate buffered saline (PBS) pH 7.2, cultures were firstly used for immuno-histochemistry. Finally, the incorporated radioactivity was counted in Ultima Gold scintillation liquid using an α,ß-scintillation analyzer (Packard 2500 TR, Packard, Groningen, NL). Radioactivity was expressed as counts per minute (cpm) and desintegrations per minute (dpm). The incorporation of BrdU was expressed as the labelling index (L.I.). The L.I. is defined as the relative number of BrdU-positive nuclei in twelve prefixed areas of 0.15 mm^2 per culture.

Pulse-chase labelling: in some experiments cells were labelled for 8 hours with 40 μg/ml BrdU in serum-free medium with the indicated growth factor. The label was chased by an excess of non-labelled thymidine in serum-free medium, and subsequently the cells were incubated with the indicated medium.

Immunohistochemistry.

After fixation of the cultures with ethanol, the BrdU and cytokeratin staining was performed as described [15]. Firstly, chain-specific cytokeratin and then BrdU expression were visualized using appropriate dilutions of the primary mouse monoclonal antibodies in a conjugated immunoenzyme assay. Secondary rabbit anti-mouse antibodies (DAKO, Etten Leur, NL) were either peroxidase-conjugated (for BrdU staining) or alkaline phosphatase-conjugated (for cytokeratin staining). As chromogens served 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Fluka, Oud Beijerland, NL) and the diazonium salt Fast red violet LB with Naphtol AS MX phosphate as coupling agent (Sigma, St. Louis, USA). As determined with RGE53 primary antibody, cytokeratin 18 was localized only in the umbrella cells of mouse urothelium in vivo (data not

shown).

To estimate the level of apoptotic cell death, nuclei were stained according to the Feulgen-reaction after visualization of the incorporated BrdU, and scored according to morphological criteria [21]. Cells were counterstained with methylgreen. A minimum of 1,000 nuclei per culture was counted in at least six prefixed areas of 0.15 mm^2.

Transmission Electron Microscopy (TEM).

Confluent cultures were rinsed with PBS pH 7.2 and fixed with 1.5% w/v glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. Next, the cultures were treated exactly as described [15]. Uranylacetate and leadnitrate contrasted ultrathin sections (0.02 μm) were studied using a transmission electron microscope (Zeiss EM902) at 80 kV.

Morphometry and image analysis.

For outgrowth studies, the edge of the outgrowth of the explant culture as visualized by a light microscope, was drawn. The area of the drawn outgrowth was measured by image analysis. Quantification of cytokeratin-stained parts of the membrane and the drawn outgrowth areas was done as described previously [15]. Cytokeratin-stained areas and outgrowth areas were expressed as percentage of the maximal outgrowth area (460 mm^2).

Statistical analysis.

All experiments were done at least three times in duplo. All data are given as the mean of all experiments ± the standard deviation. The significance of data was determined using the Student's t-test.

RESULTS

A. Model mimicking intact bladder mucosa.

Primary cultures of mouse urothelium were grown to confluency under routine culture conditions. At confluency, cultures were treated for four days with growth factors in serum-free medium. Table 1 presents the quantitative data of the parameters for growth (number of nuclei), proliferation (labelling index and thymidine incorporation), and differentiation (cytokeratin 18 expression and number of cell layers). Compared to serum-free medium alone both EGF and FGF-1 stimulated the growth and proliferation (enhanced number of nuclei, [^{3}H]-thymidine uptake, and BrdU incorporation), resulting in an

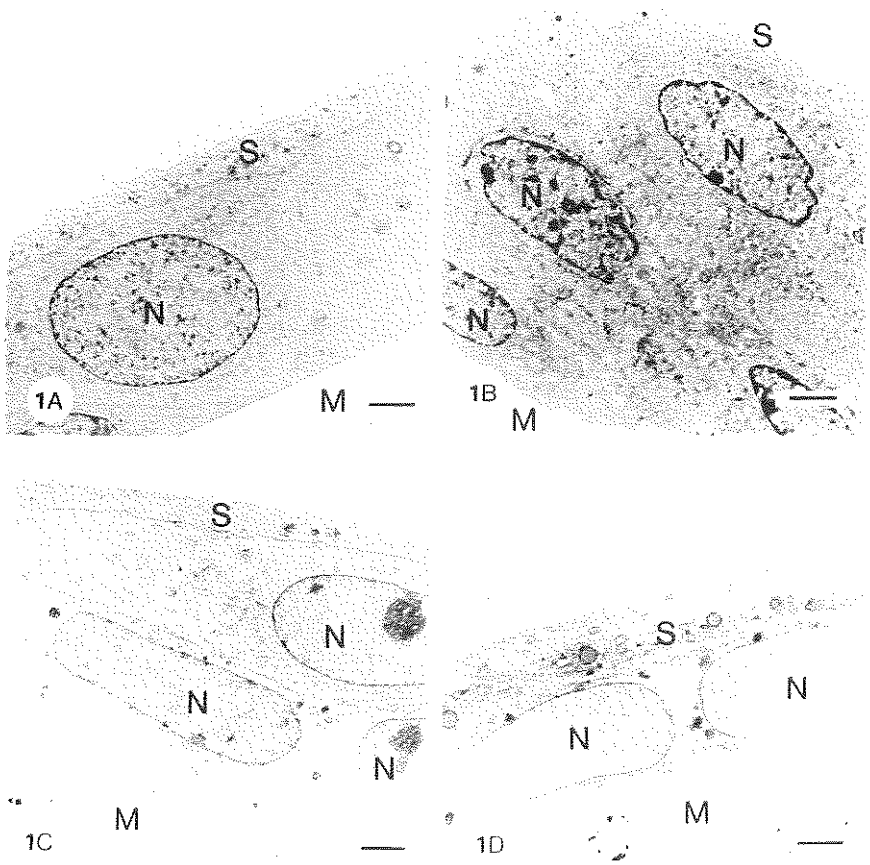

Figure 1. Transmission electron micrographs of confluent urothelial cultures treated with different growth factors during 4 days. Cultures were treated with serum-free medium alone (A), EGF (B), FGF-1 (C), or 0.5 ng/ml TGFß$_1$ (D). N = nucleus, M = membrane, S = superficial cell. Original magnification: 3000 X; bar = 2.5 μm.

increase of cell layers from 2-3 up to 6 layers as confirmed by TEM (Figure 1). The area of cells which stained for cytokeratin 18, which is considered to be a marker for terminal differentiation into superficial cells [22], is larger in FGF-1 treated cultures (Table 1). We noted that not all cytokeratin 18 positive cells have the specific morphology of the superficially located, large polygonal umbrella cells. These umbrella cells are only occasionally noted in FGF-1 or EGF treated cultures and they often exhibited a spindle cell-like morphology (Figure 2). These data indicate that exposure to EGF and FGF-1 disturbs the normal differentiated stratification of the organoid-like cultures. In contrast, both low (0.5 ng/ml) and high (2-4 ng/ml) concentrations of TGFß inhibited the proliferation

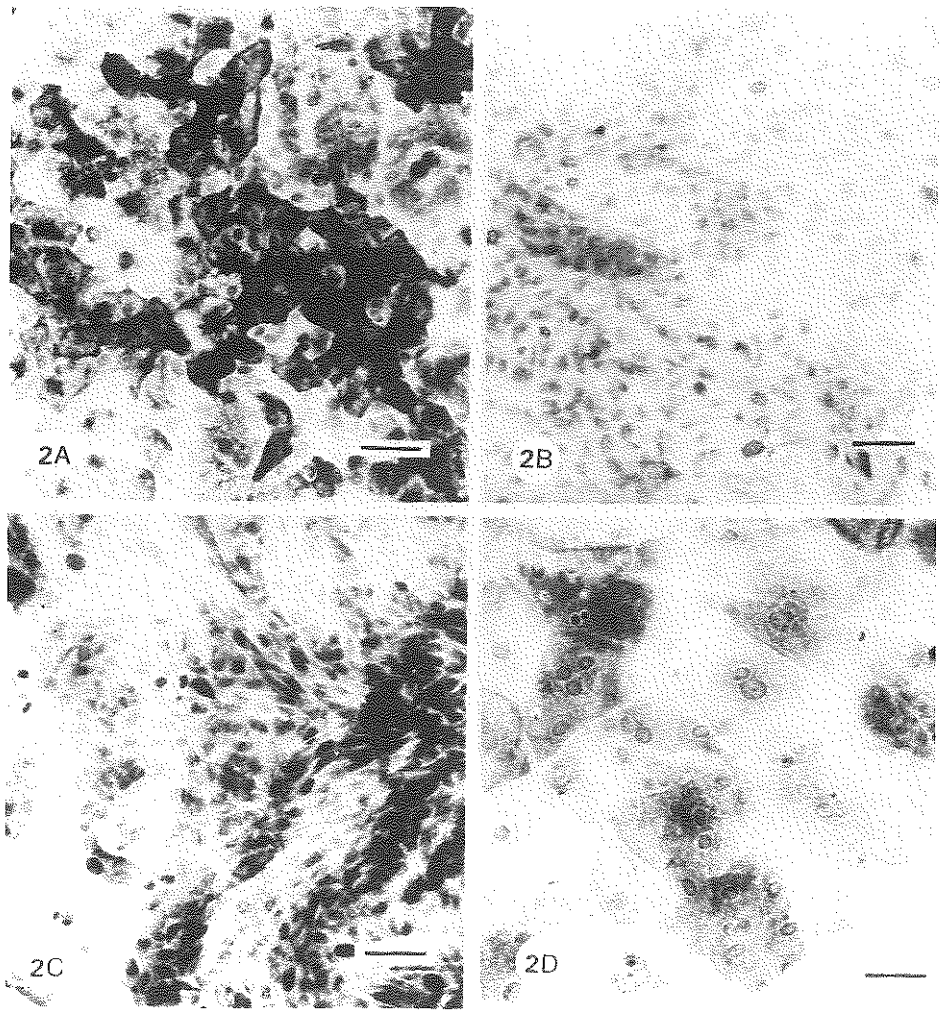

Figure 2. Micrographs of cytokeratin 18 positive, superficial urothelial cells in confluent cultures treated with different growth factors during 4 days. Cultures were counterstained with haematoxylin. Note the differences in morphology of the superficial cells. Cultures were treated with serum-free medium alone (A), EGF (B), FGF-1 (C), or 0.5 ng/ml TGFß$_1$ (D). Original magnification: 200 X; bar = 50 μm.

(lower [^{3}H]-thymidine uptake, less BrdU positive cells, and a decreased nuclear density). The number of cell layers also decreased to one. Occasionally, a second degenerating cell layer was present as observed by TEM (Figure 1). The cytokeratin 18 positive area in TGFß-treated cultures was larger compared to serum-free cultures. The superficial cells were all large with a regular polygonal

Table 1. Effects of growth factors on confluent primary urothelial cultures.

GROWTH FACTOR	NUCLEI	L.I.	[^{3}H]-THY	RGE53$^+$ AREA	CELL LAYER
serum-free	212 ± 49	1.0 ± 0.7	3411 ± 1785	32.1 ± 24.2	1-3
EGF	377 ± 59	5.5 ± 3.3	9986 ± 2393	51 ± 7.0	3-6
FGF-1	356 ± 76	3.6 ± 0.6	7372 ± 1093	72.2 ± 1.3	3-6
FGF-2	207 ± 57	1.1 ± 1.1	4839 ± 1822	54.2 ± 23.5	1-3
TGFß (0.5)	124 ± 25	0.1 ± 0.1	917 ± 749	85.3 ± 4.8	1-2
TGFß (2.0)	42 ± 23	0.0 ± 0.0	413 ± 14	73.6 ± 7.2	1
PDGF-AA	221 ± 33	1.4 ± 0.9	7954 ± 1261	33.1 ± 6.3	2-3
PDGF-BB	127 ± 28	1.3 ± 0.6	6332 ± 1533	78.3 ± 6.1	2-3
serum	264 ± 73	1.8 ± 1.5	11061 ± 1747	42.7 ± 19.1	2-3

Table 1. Effect of growth factors on proliferation and differentiation of confluent primary urothelial cultures. Cultures were incubated with the indicated growth factors for 4 days in serum-free medium alone. The table includes the number of nuclei per 0.15 mm^2, the labelling index (L.I.), the incorporation of [^{3}H]-thymidine (in dpm), the cytokeratin 18 positive area (RGE 53$^+$) related to the total growth area (460 mm^2), and the number of cell layers. The TGFß$_1$ concentrations are 0.5 or 2.0 ng/ml (indicated between brackets).

morphology resembling umbrella cells (Figure 2). These observations suggest that TGFß induced a terminal differentiation into umbrella cells (Table 1). FGF-2 did not affect any of the examined parameters. A lower dose of 10 ng/ml FGF-2 was also not effective (data not shown). Although PDGF-AA and PDGF-BB doubled the thymidine uptake, the labelling indices were similar to that of cultures treated with serum-free medium alone.

To confirm that TGFß induced terminal differentiation, we primed confluent cultures with EGF in order to increase the number of cells with the morphology of basal cells. These cultures were then pulse-chase labelled with BrdU (see materials and methods). The cultures were subsequently treated with either TGFß or serum-free medium alone. Cross sections demonstrated that the number of cell layers in the TGFß treated cultures was diminished to one or two (Figure 3A and 4), while serum-free medium treated cultures were still stratified with at least three cell layers (Figure 3B and 4). Compared with non-TGFß treated cultures, the number of non-BrdU-labelled nuclei was decreased in all cell layers in TGFß treated cultures, while the number of BrdU-labelled nuclei remained unchanged (Figure 4). Finally, the cytokeratin 18 positive area in TGFß-treated cultures (80.3% ± 0.7%) was higher than in serum-free medium alone treated cultures (71.3% ± 1.7%). In addition, the superficial cells assumed the morphology of umbrella cells only in the TGFß-treated cultures. The data given in figure 4 show that BrdU was incorporated in the originally

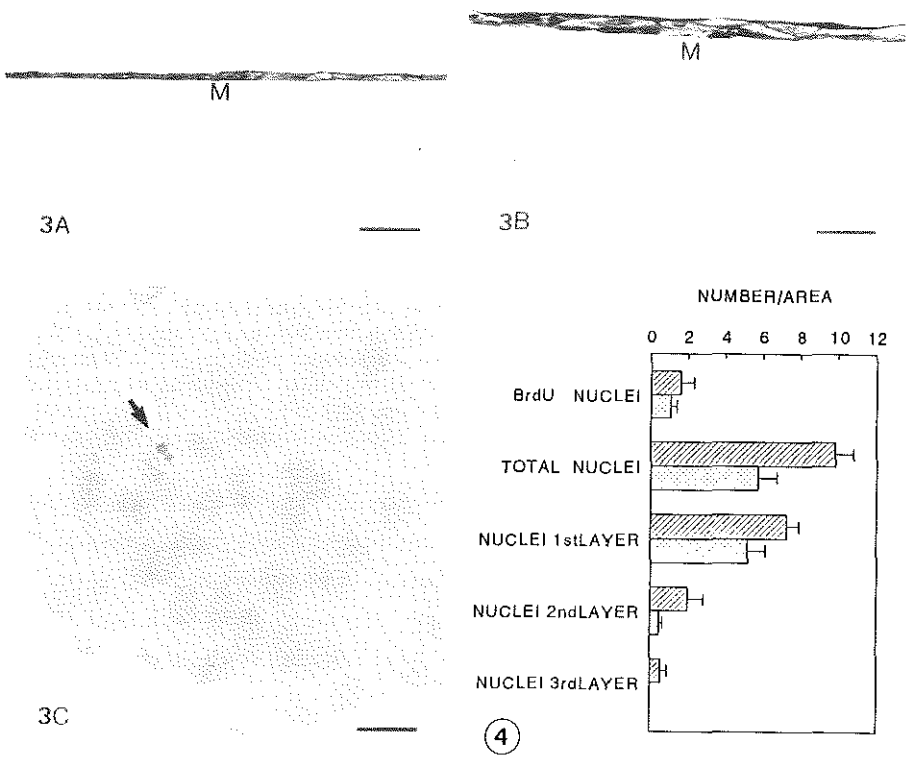

Figure 3. Induction of terminal differentiation and apoptosis by TGFß$_1$. (A,B) Cross sections of paraffin-embedded, haematoxylin-stained, and cytokeratin 18 positive, urothelial cultures treated with either 0.5 ng/ml TGFß$_1$ (A) or serum-free medium alone (B) during 6 days after pretreatment with EGF. The cytokeratin 18 positive cells are darkly stained. M = membrane. Original magnification: 400 X; bar = 25 μm. (C) Micrograph of Feulgen- plus BrdU-stained urothelial cultures treated with TGFß$_1$. The arrow indicates an apoptotic, BrdU-stained body. Original magnification: 500 X; bar = 20 μm.

Figure 4. TGFß$_1$-effect on differentiation of confluent urothelial cultures. The number of nuclei counted on cross sections of cultures treated with TGFß$_1$ (0.5 ng/ml)(stippled bars) or without TGFß$_1$ (hatched bars) is given on the X-axis. The number of nuclei was counted per cell layer on half of a cross-sectioned membrane or, as with BrdU labelled nuclei, is given for all cell layers together per half of a cross-sectioned membrane. Data are given ± standard deviation (n = 6).

basally located cell layers. So, TGFß-mediated loss of cells occurs from the superficially located cell layers. Both the induction of cell shedding, the

increased cytokeratin 18 expression, and the adoption of the morphology of umbrella cells in the superficial cell layer upon TGFß treatment can be regarded as a phenomenon of terminal differentiation.

Furthermore, TGFß has been reported to induce apoptosis in uterine epithelium [23], hepatocytes [24], and gastric carcinoma cells [25]. In the above described the model for terminal differentiation induction, we examined whether exposure to TGFß increased apoptosis. Apoptotic bodies were defined morphologically by fragmented nuclei with an intact cell membrane and a shrunken cytoplasm. Figure 3C shows apoptotic bodies in cultures in the presence of TGFß. The ratio of apoptotic bodies versus normal appearing cells was 1 out of 50 nuclei in cultures treated for 6 days with TGFß versus 1 out of 1000 nuclei in cultures treated for 6 days with serum-free medium alone.

B. Model mimicking regenerating bladder mucosa.

Table 2. Effects of growth factors on non-confluent primary urothelial cultures.

GROWTH FACTOR	NUCLEI	L.I.	[^{3}H]-THY	RGE53$^+$ AREA
serum-free	107 ± 45	2.9 ± 2.7	3485 ± 2029	67.3 ± 15.9
	183 ± 48	3.7 ± 3.7		
EGF	260 ± 52	5.7 ± 3.3	100248±46746	62.4 ± 13.6
	452 ± 49	8.1 ± 1.8		
FGF-1	239 ± 68	2.7 ± 1.6	5634 ± 1490	64.9 ± 8.9
	455 ± 111	9.2 ± 2.2		
FGF-2	116 ± 14	0.9 ± 0.5	1792 ± 11	65.1 ± 10.4
	206 ± 26	1.0 ± 0.9		
TGFß	57 ± 5	0.0 ± 0.0	194 ± 35	90.5 ± 6.5
	89 ± 34	0.0 ± 0.0		
PDGF-AA	147 ± 40	2.1 ± 2.1	1833 ± 1138	78.7 ± 14.3
	231 ± 28	1.0 ± 0.3		
PDGF-BB	126 ± 33	2.0 ± 2.0	N.D.	75.2 ± 7.8
	174 ± 18	4.6 ± 2.8		

Table 2. Effect of growth factors on proliferation and differentiation of non-confluent primary urothelial cultures. Cultures were incubated with the indicated growth factors for 4 days in serum-free medium alone. The table includes the number of nuclei per area and the labelling index (see legend of table 1) at the edge of the outgrowth (upper row) and at the number at the central part of the outgrowth (lower row). Also, the incorporation of [^{3}H]-thymidine (in dpm) and the cytokeratin 18 positive area relative to the outgrowth area (RGE53$^+$) are given. The TGFß$_1$ concentration was 0.5 ng/ml. N.D. = not determined.

Non-confluent cultures covering 55% - 65% of the maximal outgrowth area were treated for the indicated period with growth factors. The effects of the growth factors on the outgrowth area are given in Figure 5A. Treatment with 0.5 ng/ml TGFß resulted in a reduction of the outgrowth area, while treatment with FGF-1 and EGF increased this area. FGF-2 had no effect compared with serum-free medium alone. The effects of PDGF-AA or PDGF-BB on the outgrowth area are marginal. Table 2 demonstrates that treatment with either FGF-1 or EGF led to an increase in nuclear density and [^{3}H]-thymidine uptake. The FGF-1 dependent increase in [^{3}H]-thymidine uptake seemed to be transient, since treatment for 2 days led to a 5 times higher [^{3}H]-thymidine uptake (data not shown) instead of 1.5 times after a period of 4 days (Table 2). Cytokeratin 18 expression was not affected by FGF-1 or EGF. In contrast, in the presence of TGFß the number of nuclei and the thymidine uptake decreased, but the cytokeratin 18 expression and the number of umbrella cells increased.

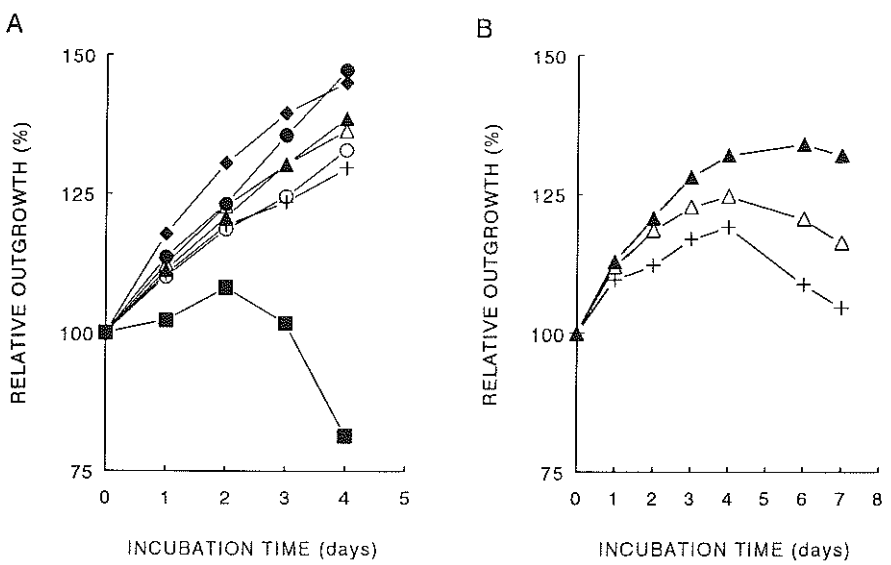

Figure 5. Effect of growth factors on the increase in the outgrowth of non-confluent urothelial cultures. When the cultures covered 55% to 65% of the maximal outgrowth area the incubation started (day 0); this outgrowth area was set at 100%. The X-axis indicates the incubation time in days, the Y-axis the relative increase in outgrowth. (A,B) Cultures with (A) or without (B) the original explant present during incubation. TGFß$_1$ concentration was 0.5 ng/ml. (+) = serum-free alone; (●) = EGF; (◆) = FGF-1; (○) = FGF-2; (Δ) = PDGF-AA; (▲) = PDGF-BB; (■) = TGFß$_1$.

Treatment with FGF-2, PDGF-AA, or PDGF-BB had no effect over serum-free medium alone.

Effects of exogenously added PDGF might be veiled by stromal-derived PDGF. In our cultures, a small remnant of the stromal component was generally left as the inoculated explant remained attached during culture. In order to exclude the potential influence of stromal-derived PDGF or FGF-2, we removed the explant at 55% - 65% of confluency, and treated the cultures during 7 dayswith or without PDGF-AA, PDGF-BB, or FGF-2 in serum-free medium. Figure 5B shows the effect of the PDGF's on the outgrowth area, revealing that the outgrowth area was significantly enhanced in the presence of PDGF-BB ($P < 0.02$ at days 3 to 7) but not in the presence of PDGF-AA. We did not observe any effect of FGF-2 on the outgrowth area (data not shown).

DISCUSSION

In the present study direct actions of various growth factors on urothelium with respect to several parameters which were investigated simultaneously on a single primary, organoid-like urothelial culture. The qualitative effects of these growth factors in the model representing the intact urothelium and in the model mimicking regenerating urothelium were similar. Both EGF and FGF-1 stimulated the proliferation, causing 1) a higher nuclear density reflecting an increase in the number of cells and 2) a multilayering of cells associated with a lack of differentiation of the stratified epithelium. Instead, TGFß 1) inhibited proliferation, 2) induced (terminal) differentiation, and 3) induced apoptosis, resulting in a decrease in cell number and cell layers. The effects of FGF-2, PDGF-AA, and PDGF-BB on primary cultures of transitional epithelial cells were minimal with respect to the examined parameters. A slight, but significantly enhanced urothelial expansion was observed in the presence of PDGF-BB, but not PDGF-AA, in the regeneration model in the absence of stromal cells (Figure 5).

Proliferation and migration

EGF and FGF-1 have been shown to induce the proliferation and migration of epithelial cells, including urothelial cells, in vitro [6,16,26,27]. FGF-2 also stimulated the proliferation but not the migration, of urothelial cell lines [16,17]. In vivo, EGF is present in the urine. FGFs are present e.g. in endothelial

basement membranes and can be released upon injury. Furthermore, FGF-1 but not FGF-2 mRNA is highly expressed in regenerating mouse urothelium in vivo [De Boer et al, submitted]. These studies and the present data on EGF and FGF-1 obtained with an organoid-like epithelial culture support the hypothesis that after injury EGF-like growth factors and FGF-1 stimulate the re-epithelialization of urothelium in vivo as seen for skin epithelium [28,29]. This may be achieved directly by inducing both the proliferation and the migration of epithelial cells forming a stratified epithelium. Furthermore, FGF-1 seems to be a more important regulatory factor than FGF-2 in the physiology of mouse bladder epithelium.

Some recent studies suggested an autocrine mechanism for PDGF in epithelial cells during wound healing [2] and in carcinomas [30,31]. Only a few studies reported on a biological function of PDGF on epithelial cells in vitro, e.g. induction of the proliferation of urothelial cell lines [16] or the maturation of lens epithelium [32]. Induction of epithelial migration has previously been reported for retinal epithelial cells [33] and keratinocytes [34]. The present study demonstrated a slight induction of urothelial expansion by PDGF-BB and not PDGF-AA suggesting a functional difference between these two PDGFs in urothelium. Though, the biological role of PDGF on epithelia in vivo is not yet clear.

We failed to observe an effect of TGFß on migration of transitional epithelial cells in contrast to studies on keratinocytes [19,35]. Nickoloff et al [35] observed that human keratinocytes in vitro migrated after a 2-days pretreatment with 2—20 ng/ml TGFß$_1$. Upon a 3 to 5 days treatment of confluent cultures with 2 or 4 ng TGFß$_1$/ml serum-free medium the outgrowth area decreased due to terminal differentiation. After a 2-days treatment with 0.5 ng TGFß$_1$/ml serum-free medium, we also noted a decrease in the outgrowth area of non-confluent cultures. According to Nickoloff et al [35] only undifferentiated cells are able to migrate. This may explain the lack of migration in response to TGFß$_1$ in our experiments since TGFß$_1$ induced terminal differentiation in our cultures. Hebda [19] observed that TGFß induced the migration of keratinocytes in the first three days of a skin explant culture. In these experiments TGFß might have affected the migration of only basal (undifferentiated) cells.

Differentiation

Data from this study demonstrated that TGFß both inhibits the proliferation and induces the terminal differentiation of transitional epithelial cells

into cells with the morphology and cytokeratin expression of umbrella cells. Limited numbers of umbrella cells are also present in untreated cultures. The data support the observations of Glick et al [36] and Sacco et al [14] on TGFß-

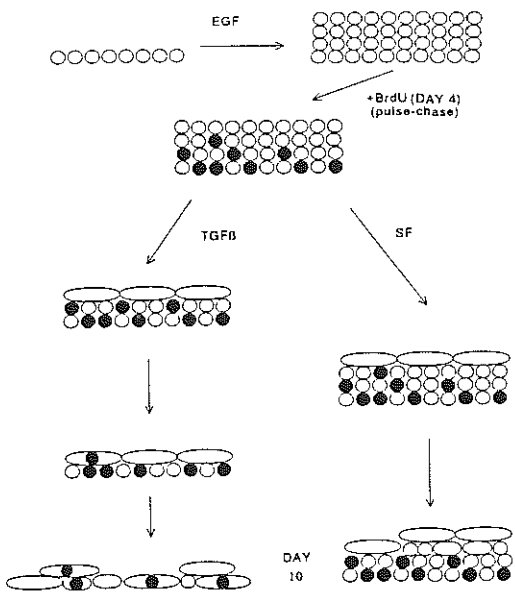

Figure 6. Schematic diagram depicting the subsequent action of growth factors on proliferation and differentiation of transitional epithelial cells, resulting from the terminal differentiation induction experiments. Circles, ovals, and flattened ovals represent undifferentiated cells, intermediate differentiated cells, and differentiated umbrella cells, respectively. Closed symbols represent BrdU-labelled cells.

induced terminal differentiation of keratinocytes and bronchiocytes in primary culture. Two modes of action for TGFß can be proposed. Firstly, TGFß may induce the upper cell layer to differentiate terminally into umbrella cells and to shed. Alternatively, TGFß may induce the basally and intermediate located cells to differentiate, while terminal differentiation and shedding occur also in the absence of TGFß. From our data we cannot conclude which mechanism prevailed in our cultures. Though, TGFß induced mainly a decrease in the number of cells in the upper two cell layers, while in the pulse-chase-labelling experiment the overall number of BrdU-labelled cells remained unchanged (Figure 4). Furthermore, in TGFß-treated cultures superficial cells showed the typical appearance of umbrella cells. Hence, it seems more probable that TGFß induced terminal differentiation of superficial cells, which favours the first hypothesis. This mode of action is depicted in Figure 6.

The data of the present study also demonstrate that TGFß induced apoptosis in transitional epithelial cells. This finding is in line with observations with epithelial cells from uterus [23], liver [24], and a gastric carcinoma [25]. Our results are consistent with a biological function of TGFß in transitional cell epithelium in vivo. TGFß is present in the urine and in normal urothelial cells [8,11,12], and upon injury, TGFß is released by blood platelets and activated macrophages. Moreover, both the $TGF\beta_1$ and TGFß type II receptor protein expression is enhanced in differentiating urothelium during wound healing of the mouse bladder [De Boer et al, submitted]. Hence, the physiological maintenance of existing or regenerating transitional epithelium may be regulated by TGFß through the induction of terminal differentiation of transitional epithelium resulting in an organoid-like stratification of the epithelium. This seems to be analogous to regenerating skin epithelium in which $TGF\beta_1$ is present especially during the differentiation phase of the epidermis [37]. TGFß may also repress hyperplastic or excessive transitional cell growth after injury by inhibiting the proliferation and by induction of apoptosis within the urothelium.

Neither EGF nor FGF-1 induced the terminal differentiation of transitional epithelial cells under the present conditions. Instead, these growth factors induced a spindle cell-like morphology of the cells, while cells with the morphology of umbrella cells were rarely seen. Several of the superficial spindle cell-like cells stained immunohistochemically for cytokeratin 18, in contrast to earlier studies reporting that cytokeratin 18 staining with the RGE53 antibody was confined only to umbrella cells [22]. The spindle cell-like morphology was also noted in other studies with urothelial cells treated with EGF or TGFα [27,38], and in the rat NBT-2 cell line treated with FGF-1 [17]. The phenotypic change was associated with the induction of characteristics of transformed cells [27,38]. In patients with TCC the urothelium adjacent to the TCC often show characteristics of transformed cells which is considered to be a preneoplastic lesion [39]. Since both TGFα and FGF-1 have been found in TCC or in urine of patients with TCC [8,9,40,41], the possibility should be considered that this transformed phenotype of the urothelium may be caused by these tumour-derived growth factors rather than reflecting a preneoplastic lesion.

In the present study we have characterized direct effects of different growth factors on primary mouse urothelial cultures which closely resemble mouse urothelium in vivo. We demonstrated distinct functions for these growth factors in the physiology of murine transitional epithelial cells in vitro. The data suggest that EGF and FGF-1 serve as proliferation and migration factors for

transitional epithelial cells in physiological processes. PDGF-BB may act as a migration factor for transitional epithelial cells under specific conditions, and TGFß acts as a differentiation factor. We did not address the complexity of growth factor interactions between similar and different cell types in this study. Furthermore, a recent study on skin wound healing suggested that FGF-7 is also an important factor in epithelial regeneration [42]. Therefore, the importance of other growth factors for the urothelial physiology should also be considered. Future investigations using this culture method should provide data on direct actions of combinations of growth factors on the urothelium and on stroma-urothelium interactions under physiological and pathological conditions.

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REFERENCES

1 Cross M, Dexter TM. Cell 64, 1991:271-280.

2 Antoniades HN, Galanopoulos T, Neville-Golden J, Kiritsy CP, Lynch SE. Am. J. Pathol. 142, 1993:1099-1110.

3 Pusztai L, Lewis CE, Lorenzen J, McGee JO'D. J. Pathol. 169, 1993:191-201.

4 Aaronson AS. Science 254, 1991:1146-1153.

5 Thompson NL, Flanders KC, Smith JM, Ellingsworth LR, Roberts AB, Sporn MB. J. Cell Biol. 108, 1989:661-669.

6 Zhang G, Ichimura T, Wallin A, Kan M, Stevens J. J. Cell. Physiol. 148, 1991: 295-305.

7 Carpenter G. J. Cell Sci. Suppl. 3, 1985:1-9.

8 Kimball ES, Bohn WH, Cockley KD, Warren TC, Sherwin SA. Cancer Res. 44, 1984:3613-3619.

9 Chodak GW, Hospelhorn V, Judge SM, Mayforth R, Koeppen H, Sasse J. Cancer Res. 48, 1988:2083-2088.

10 Floege J, Johnson RJ, Alpers CE, Fatemi-Nainie S, Richardson CA, Gordon K, Couser WG. Am. J. Pathol. 142, 1993:637-650.

11 Kawamata H, Azuma M, Kameyama S, Nan L, Oyasu R. Cell Growth Differ. 3,

1992:819-825.

12 Coombs LM, Pigotti DA, Eydmann ME, Proctor AJ, Knowles MA. Br. J. Cancer 67, 1993:578-584.

13 Wilke MS, Hsu BM, Wille JJ, Pittelkow MR, Scott RE. Am. J. Pathol. 131, 1988:171-181.

14 Sacco O, Romberger D, Rizzino A, Beckmann JD, Rennard SI, Spurzem JR. J. Clin. Invest. 90, 1992:1379-1385.

15 De Boer WI, Rebel JMJ, Thijssen CDEM, Vermey M, van der Kwast TH. J. Histochem. Cytochem. 42, 1994:277-282.

16 De Boer WI, Rebel JMJ, Foekens JA, Vermey M, van der Kwast TH. Int. J. Cancer 54, 1993:1022-1027.

17 Vallés AM, Boyer B, Badet J, Tucker GC, Barritault D, Thiery JP. Proc. Natl. Acad. Sci. (USA) 87, 1990:1124-1128.

18 Dubeau L, Jones PA. Cancer Res. 47, 1987:2107-2112.

19 Hebda PA. J. Invest. Dermatol. 91, 1988:440-445.

20 Hirata Y, Orth DN. Endocrinology 105, 1979:1383-1387.

21 Kerr JFR, Wyllie AH, Currie AR. Br. J. Cancer 26, 1972:239-257.

22 Herman CJ, Vegt PDJ, Debruyne FMJ, Vooijs GP, Ramaekers FCS. Am. J. Pathol. 120, 1985:419-426.

23 Rotello RJ, Lieberman RC, Purchio AF, Gerschenson LE. Proc. Natl. Acad. Sci. (USA) 88, 1991:3412-3415.

24 Oberhammer FA, Pavelka M, Sharma S, Tiefenbacher R, Purchio AF, Bursch W, Schulte-Hermann R. Proc. Natl. Acad. Sci. (USA) 89, 1992:5408-5412.

25 Yanagihara K, Tsumuraya M. Cancer Res. 52, 1992:4042-4045.

26 Barrandon Y, Green H. Cell 50, 1987:1131-1137.

27 Yura Y, Hayashi O, Kelly M, Oyasu R. Cancer Res. 49, 1989:1548-1553,

28 Lynch SE, Colvin RB, Antoniades HN. J. Clin. Invest. 84, 1987:640-646.

29 Mellin TN, Mennie RJ, Cashen DE, Ronan JJ, Capparella J, James ML, Disalvo J, Frank J, Linemeyer D, Giminez-Gallego G, Thomas KA. Growth Factors 7, 1992:1-14.

30 Heldin N-E, Cvejic D, Smeds S, Westermark B. Endocrinology 129, 1991:2187-2193.

31 Antoniades HN, Galanopoulos T, Neville-Golden J, O'Hara CJ. Proc. Natl. Acad. Sci. (USA) 89, 1992:3942-3946.

32 Brewitt B, Clark JI. Science 242, 1988:777-779.

33 Campochiaro PA, Glaser BM. Arch. Opthalmol. 103, 1985:576-579.

34 Esienger M. Epidermal derived factors. Third International Wound Healing Symposium, Miami. 1990.

35 Nickoloff BJ, Mitra RS, Riser BL, Dixit VM, Varani J. Am. J. Pathol. 132, 1988: 543-551.

36 Glick AB, Danielpour D, Morgan D, Sporn MB, Yuspa SH. Mol Endocrinol 4, 1990:46-52.

37 Levine JH, Moses HL, Gold LI, Nanney LB. Am. J. Pathol. 143, 1993:368-380.

38 Knowles MA, Hicks RM, Dave H, Harvey AE, Roberts AB, Sporn MB. Carcinogenesis 6, 1985:595-604.

39 Koss LG. *in* Tumors of the urinary bladder (Firminger, H.I., Ed.), Vol. 11, Armed Forces Institute of Pathology, Washington. 1975.

40 Ravery V, Jouanneau J, Gil Diez S, Abbou CC, Caruelle JP, Barritault D, Chopin DK. Urol Res 20, 1992:211-214.

41 Chopin DK, Caruelle JP, Colombel M, Palcy S, Ravery V, Caruelle D, Abbou CC, Barritault D. J. Urol. 150, 1993:1126-1130.

42 Werner S, Peters KG, Longaker MT, Fuller-Pace F, Banda MJ, Williams LT. Proc. Natl. Acad. Sci. USA 89, 1992:6896-6900.

Chapter 4.

EXPRESSION OF GROWTH FACTORS AND RECEPTORS DURING SPECIFIC PHASES IN REGENERATING UROTHELIUM AFTER ACUTE INJURY IN VIVO.

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ABSTRACT

We investigated the spatio-temporal changes in RNA and protein expression of growth factors and their receptors by in situ hybridization and immunocytochemistry during regeneration after acute injury of mouse urothelium in vivo. These data were correlated with changes in morphology and proliferation during regeneration. Except for an enhanced muscular TGFß$_1$ and TGFß type II receptor expression, changes in expression patterns of growth factors or receptors were confined to the urothelium. Increased mucosal RNA expression of IGF-II and particularly of type I IGF receptor, as well as FGF-1, but not of FGF-2, coincided with re-epithelialization and urothelial proliferation. Both high level of urothelial TGFß$_1$ RNA and protein expression were associated with re-epithelialization and differentiation. In addition, TGFß type II receptor protein expression was similarly enhanced in the same urothelial cells. PDGF-A RNA was expressed constitutively in the mucosa but decreased in the re-epithelialization phase. The data are consistent with the notion that urothelial regeneration can be achieved by paracrine or autocrine acting, urothelium-derived growth factors. Since analogous growth factor RNA expression patterns in regenerating skin epidermis have been found, a more general growth factor-regulated mechanism for epithelial regeneration may be suggested.

INTRODUCTION

Bladder urothelium consists of a three-layered transitional epithelium. Under normal conditions, this slowly proliferating epithelium has a very low turn-over (1). Upon urothelial injury e.g. by catheterization, endoscopical examination, biopsies, deposition of urinary crystals, acute outlet obstruction, or short exposure to chemicals, an enormous increase in urothelial proliferation is observed resulting in a rapid re-epithelialization (1-4). Other organs like stomach and gall bladder also show a rapid re-epithelialization of the mucosa layer upon damage (5,6). Such a fast epithelialization results in a rapid restoration of the functional integrity of these epithelia. Little is known about the factors involved in this process. Studies on skin epithelium suggested that multiple growth factors and growth factor binding proteins are implicated in wound healing of the skin epithelium. These growth factors may be derived from e.g. damaged blood platelets, endothelial basement membranes, and activated, infiltrating macrophages, but also from epithelial cells. Among these growth factors are

epidermal growth factor (EGF), transforming growth factor ß (TGFß), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) (7-14). Recent studies also demonstrated FGF-1 protein expression in regenerating stomach epithelium (15) and an acceleration of gastric ulcer healing by FGF-1 application in vivo (16).

Direct actions of growth factors on urothelial cells include stimulation of [^{3}H]-thymidine uptake by basal urothelial cells in vivo (17), stimulation of proliferation and migration of murine urothelial cell lines and urothelial cells in primary culture by EGF and FGF-1 (18-21), inhibition of proliferation and induction of terminal differentiation of mouse urothelial cell lines or urothelial cells in primary culture by TGFß$_1$ (20,21). Furthermore, cell cycle progression factors like IGF-I and IGF-II, are also required for growth of epithelial cells, including urothelial cells (20), while the IGF-mediated actions may be affected by insulin-like growth factor binding proteins (IGFBPs) (22,23). Little is known about PDGF action on epithelial cells. Few studies report a biological function of PDGF in epithelia. Antoniades et al (11,12) demonstrated that during regeneration porcine skin epithelial cells express both PDGF and PDGF receptors. Expression of PDGF type α receptor was found in rat olfactory epithelium (24) and in three murine urothelial cell lines (20). PDGF was shown to enhance growth and maturation of lens epithelium in vitro (25), to stimulate the proliferation of mouse urothelial cell lines (20), and to induce migration of retinal pigment epithelial cells (26).

Few growth factors like TGFß and EGF, normally occur in the urine (27,28). In addition, normal urothelial cells are reported to express TGFß RNA in vivo (29,30). Upon injury to skin dermis and epidermis, several growth factors like TGFßs, FGFs, IGFs, and PDGFs are released by both mesenchymal cells (e.g. blood platelets, endothelial cells, and activated, infiltrating macrophages) and epithelial cells. In analogy to skin epidermis, it may be hypothesized that these growth factors have distinct functions during urothelial regeneration after injury. Synthesis, expression, and functioning of most growth factors in transitional bladder epithelium has not yet been established during wound healing in vivo. In this study we correlated the expression of different growth factors and growth factor receptors at the RNA or the protein level in regenerating urothelium with proliferation, differentiation, and migration of urothelial cells. We used mice in which we shortly overdistended the urine bladder causing acute superficial damage to the urothelium, but little damage to the submucosa and muscle layer. At several intervals after the injury, bladders

were dissected for determination of growth factors and growth factor receptor RNA or protein using in situ hybridization and immunocytochemical techniques, respectively.

MATERIALS AND METHODS

Chemicals

RNA polymerases, tRNA, and RNAse inhibitor were obtained from Pharmacia (Woerden, NL). RNAse-free DNAse was purchased from Promega (Madison, USA), and RNAse T1 from Boehringer (Mannheim, Germany). cDNA probes for mouse IGF-I and mouse IGF-II were kindly provided by Dr. G.I. Bell, the rat type I IGF receptor by Drs. H. Werner and D. LeRoith, and the human PDGF-A and PDGF type α and type ß receptor by Dr. C.-H. Heldin. Anti BrdU antibody IIB5 and anti TGFß$_1$ antibody were kindly donated by Dr. F.C.S. Ramaekers and Dr. W. Boersma. Rabbit polyclonal antibodies against TGFß type II receptor was purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

Bladder injury experiments

Female BCBA mice 8 to 10 weeks of age were housed in macrolon cages and fed ad libitum with water and murine chow food (Hope Farms, Woerden, NL). The bladder was distended by intravesical injection of 200 μl of 0.15 M NaCl via a catheter. This overstretching was maintained for 5 minutes. Histological examination of the kidneys did not reveal any damage due to reflux of the urine/NaCl. The presence of blood in the urine immediately after the treatment was a qualitative measure for the presence of lesions in the urine bladder. Control mice did not undergo this treatment. After overdistension of the bladder, the mice were kept under the mentioned caging conditions for one hour to 10 days. Before mice were sacrificed by CO_2 aneasthesia, mice received intraperitonially BrdU in 0.15 M NaCl (40 mg/kg body weight) during one hour. Six mice per interval were sacrificed. Isolated bladders were fixed in 4% phosphate-buffered formalin during 16 hours before embedding in paraffin.

cRNA probe generation and labelling

cDNA fragments specific for the used growth factors or receptors were ligated into pBluescript KS +/- (Stratagene, La Jolla, USA). Labelled RNA transcripts were obtained from linearized plasmid using T7, T3, or SP6 RNA polymerase, respectively, in the presence of 50 μCi [^{35}S]-UTP (Amersham,

Aylesbury, UK) as described (31). For the present study we used an EcoR I-Sac I fragment of PDGF-A cDNA, a Sst II-Pvu II fragment of the PDGF-B cDNA, a Sac I-Kpn I fragment of the PDGF α receptor cDNA, a Pvu II fragment of the PDGF ß receptor cDNA, a Bgl II-Xho I fragment of the FGF-1 cDNA, an EcoR I-Acc I fragment of FGF-2 cDNA, a Sma I-BamH I fragment of TGFß$_1$ cDNA, an EcoR I fragment of mouse IGF-I cDNA, an EcoR I-Sac I fragment of mouse IGF-II cDNA, and a 265 bp EcoR I fragment of the rat IGF type I receptor cDNA. A cDNA probe of rat IGFBP-2 was generated by PCR (31).

In situ hybridization

Embedding of bladders, sectioning, and pretreatment procedures were performed as described previously (31). Sections (5 μm thick) of paraffin-embedded bladders were mounted on slides precoated with 3-amino propyltri-oxy-silane (Sigma, St. Louis, USA). After the pretreatment and subsequent dehydration with an increasing ethanol gradient and drying, the sections were prewarmed to 63°C. Sections were then hybridized with [^{35}S]-labelled RNA probes (2 X 10^5 cpm/slide) for 16 hours at 63°C in a solution containing 50% formamide, 1X Denhardt's solution, 1 mg/ml tRNA, 10% dextransulphate, 10 mM dithiothreitol, 0.2 mg/ml Hering sperm DNA, and 1X (for the PDGF-A probe) or 4X SSC (for the other probes). After the hybridization, sections were washed subsequently with 50% formamide/2X SSC at 50°C (two times); 20 mM ß-mercaptoethanol/0.1X SSC at 62°C; 1 mM EDTA/2X SSC with 2 U/ml RNAse T1 at 37°C; and 20 mM ß-mercaptoethanol/0.1X SSC at 62°C. After dehydration with increasing ethanol gradients containing 0.3 M sodiumcitrate, the sections were dried and exposed to Kodak AR X-ray film (Eastman Kodak, Rochester, USA) for 1-9 days. Finally, sections were exposed to Kodak NTB2 autoradiographic emulsion for 1 to 21 days, developed and stained with nuclear fast red for histochemical analysis. The level of non-specific binding was determined using RNA sense probes for each growth factor or receptor.

Immunohistochemistry

After removal of paraffin by xylene, sections were hydrated and before incubation with the anti BrdU antibody, treated with HCl and Borax buffer as described (32). Antigen expression was demonstrated with appropriate dilutions of the primary antibody in a conjugated immunoenzyme assay using a secondary peroxidase-conjugated rabbit anti-mouse antibody (DAKO, Etten Leur, NL). As chromogen served 3,3'-diaminobenzidine tetrahydrochloride (Fluka,

Oud-Beijerland, NL). Finally, sections were counterstained with haematoxylin and mounted in malinol. PBS instead of primary antibody was used as a control. As a control for binding of the TGFß type II receptor antibody, we preincubated this antibody with the TGFß type II receptor synthetic peptide against which the antibody was raised, as described by the manufacturer. The immunostaining disappeared after this treatment.

Analysis of RNA expression and immunohistochemistry

Successive sections of overdistended mouse bladders and non-treated mouse bladders were used for hybridization with an antisense probe, immunohistochemical analysis of BrdU expression and morphological analysis, and hybridization with the related sense probe, respectively. The level of RNA expression was determined semi-quantitatively since variations in thickness of the autoradiographic emulsions precluded an exact quantification. The level of RNA expression was compared with aspecific binding of the probe to the luminal area of the bladder sections. The sense probes did barely hybridize to the sections. The level of protein expression was also detemined semi-quantitatively.

Morphological analysis was done by light microscopy with respect to different parameters. The presence of a single cell layer of flattened, elongated cells in the wound area was considered a parameter for migrating cells. For maturation and differentiation we examined the number of cell layers and the presence of large, flattened, superficially located umbrella cells. Furthermore, we examined the extent of the damage and the repair by the presence of extra-vascular erythrocytes, oedema, and infiltrating leukocytes.

For analysis of the proliferation, the number BrdU-positive nuclei was counted among 1600 urothelial nuclei at four different, preset sites per bladder. The urothelial proliferation per bladder is expressed as the labelling index (L.I.):

$$\frac{\text{number of BrdU-positive nuclei}}{\text{total number of nuclei}} \times 100\%$$

Both the L.I. in Table 1 and the RNA or protein expression levels in Figure 3 were calculated as the mean of 6 bladders ± the standard deviation at each time point.

RESULTS

Firstly, we examined the morphology and proliferation of regenerating urothelia at different intervals after injury. As presented in Table 1, the urothelia of untreated mice are stratified in three cell layers with normal differentiation into superficial umbrella cells. The overall proliferation is very low to nihil. One

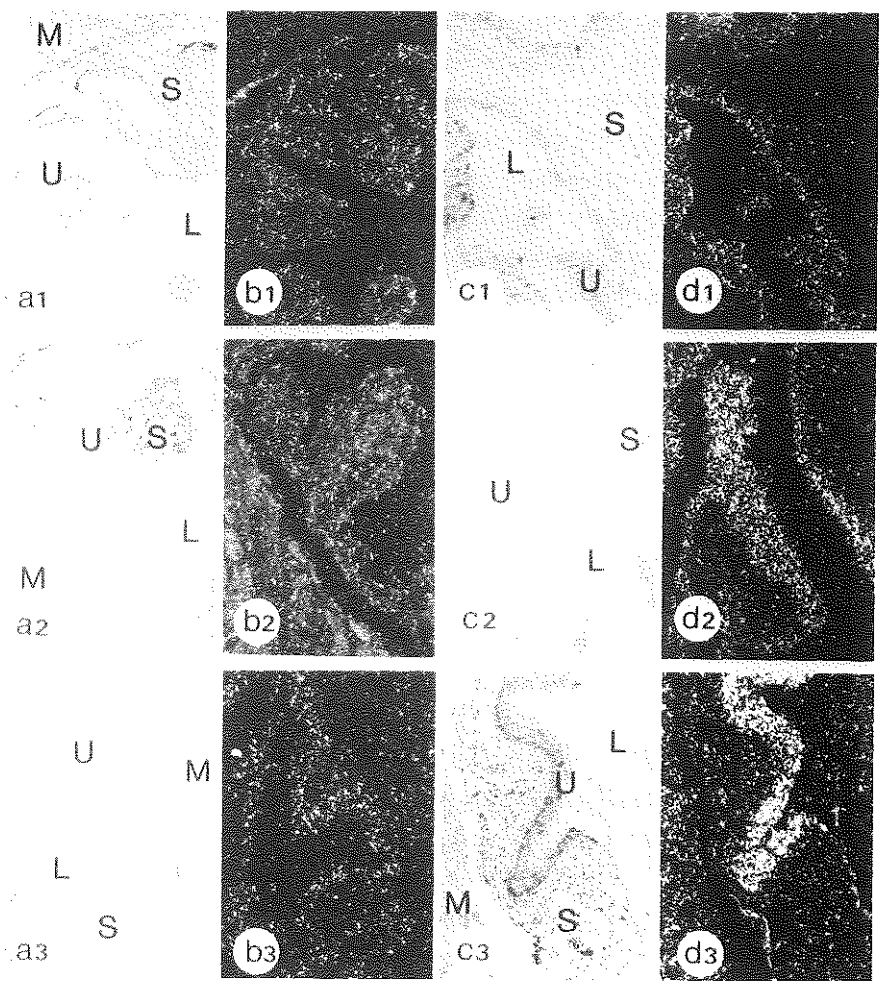

Figure 1. Localization of RNA expression in the normal, intact mouse bladder (A,B) and the regenerating mouse bladder 24 hours after injury (C,D). Micrographs A and C are bright field photos, and B and D show the corresponding dark field images. RNA localization for FGF-1, type I IGF receptor, and TGFß$_1$ is given in micrographs 1 to 3, respectively. Sections were stained with nuclear fast red. L = lumen of the bladder; U = urothelium; S = submucosa; M = muscle. Magnification = 200X.

hour after the injury, the damage to the urothelium was multifocal with frequently a complete loss of urothelium. The overall proliferation was already increased at 8 hours after the injury and was maximal at 16 hours. As early as 4 hours after the injury, a re-epithelialization was seen in wounds where the urothelium had completely detached. This process started adjacent to intact mucosa and was completed after 24 hours. After 3 days, the L.I. was decreased and the urothelia were hyperplastic. Terminally differentiated umbrella cells were noted to line hyperplastic urothelia at day 5. The proliferation was normalized after 5 to 10 days of repair and at day 10 the urothelia showed the differentiated stratification of normal, intact urothelium.

The in situ hybridization and immunocytochemistry experiments visualized the cells expressing growth factor and growth factor receptor RNA and protein, respectively. The cellular RNA localization in mouse bladders is given in Figure 1, the protein expression in Figure 2. A semi-quantification of the RNA expression levels in the urothelium during regeneration is presented in Figure 3. In untreated mouse urothelium, the expression of FGF-1, FGF-2, IGF-I, type I IGF receptor, and PDGF-B chain RNA was low to undetectable, while IGF-II, TGFß$_1$, and PDGF A-chain RNAs were expressed constitutively (Figure 1 and 3). In bladders of control mice, TGFß$_1$ RNA localized in particular to the muscle cells. Strikingly, the TGFß$_1$ RNA expression in urothelial cells was strongly enhanced within 1 hour after the injury, especially in the epithelial cells next to the site of injury (Figure 1 and 3). This expression pattern changed to an overall high expression throughout the urothelium at 8 hours after the injury, and was decreased after 3 days in hyperplastic urothelium (Figure 3 and Table 1). At day 5 when the urothelium started to show a fully differentiated stratification with superficial umbrella cells, the TGFß$_1$ RNA expression was increased again (Figure 3). The TGFß$_1$ protein expression during urothelial regeneration closely parallelled its RNA expression (Figure 2 and 3). In control bladder, a low level of TGFß$_1$ protein was noted in basal urothelial cells, while during wound healing both basal and intermediate cells were stained more intensively compared with control bladder. After 5 days, mainly the superficial cells stained for TGFß$_1$, coinciding with terminal differentiation of the superficial cells into umbrella cells. In addition, TGFß type II receptors were expressed by the same urothelial cells and its temporal expression pattern followed that of TGFß$_1$ proteins (Figure 2 and 3).

The urothelial RNA expression of PDGF-A chain and, to a lesser extent, PDGF-B chain, as well as FGF-1, but not FGF-2, was increased maximally after

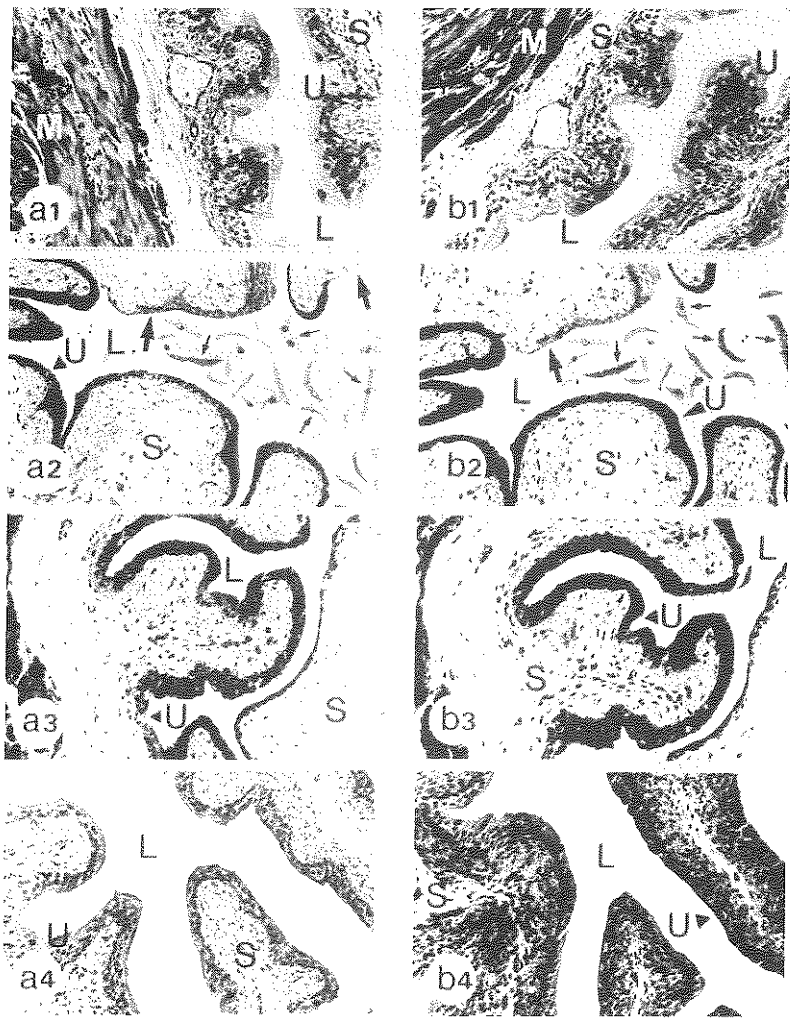

Figure 2. Immunocytochemical peroxidase-staining of TGFß$_1$ (A) and TGFß type II receptor (B) during mouse urothelial regeneration in vivo. The micrographs show: intact bladder mucosa from control mice (1), and damaged mucosa 1 hour, 24 hours, and 5 days after the injury (2,3, and 4, respectively). Sections were counterstained with haematoxylin. L = lumen of the bladder; U = urothelium; S = submucosa; M = muscle. The large arrows indicate denuded bladder submucosa. Note the detached umbrella cells in A2 and B2 (small arrows). Magnification = 200 X.

24 hours (Figures 1 and 3), coinciding with the re-epithelialization and enhanced urothelial proliferation (Table 1). Furthermore, the expression of IGF-II and type I IGF receptor was maximal after 24 hours (Figures 1 and 3), also coinciding with re-epithelialization and a maximal urothelial proliferation. In addition, we did not

observe alterations in the constitutive expression of IGFBP-2, which was mainly confined to the basal urothelial cells. Expression of IGFBP-1 and IGFBP-3 to 6 was not detectable in the bladder (data not shown). The expression of PDGF type α and type ß receptor, as well as IGF-I RNA were undetectably low in the urothelium.

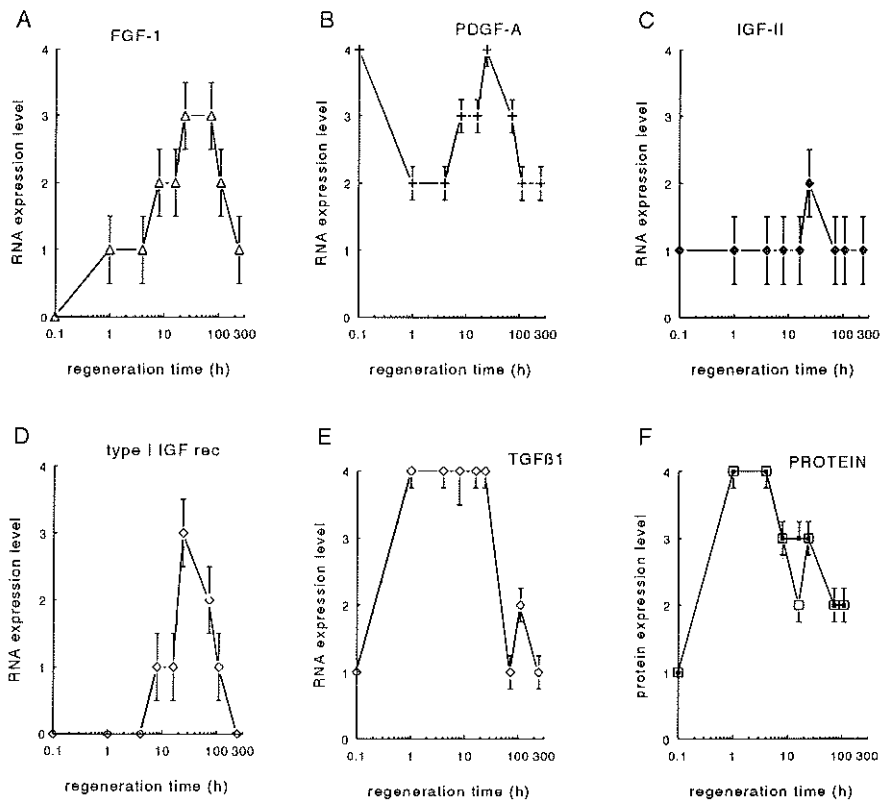

Figure 3. Semi-quantification of RNA expression levels during regeneration of the urothelium. The regeneration time after injury is given in hours. RNA expression levels of FGF-1, PDGF-A, IGF-II, type I IGF receptor, and TGFß$_1$ are given in A,B,C,D, and E, respectively. The TGFß$_1$ (open square) and TGFß type II receptor (closed square) protein expression levels are given in F. RNA and protein expression levels were examined in the wounded area of the urothelium, and calculated as the mean expression ± standard deviation (n = 6). 0 = no detectable expression; 1 = low expression; 2 = moderate expression; 3 = high expression; 4 = very high expression.

Overstretching the bladder often caused oedema of the submucosa and occassionally some haemorrhage in the muscle layer. The oedema diminshed within 5 days. Only a slight increase in the number of BrdU-positve cells was

noted in both the submucosa and the muscle layer between 24 hours and 5 days after the injury. At day 10, the proliferation and morphology of the submucosa and muscle layer was normalized (data not shown). In muscle cells from control bladder, TGFß$_1$, TGFß type II receptor, IGF-II, and, to a lesser extent, IGF-I were highly expressed, while PDGF-B RNA was not detected. The expression of TGFß$_1$ RNA and protein in the submucosa, as well as FGF-1 and PDGF-A in both muscle and stromal layers, was low in control bladder. Only the muscular TGFß$_1$ RNA and protein and TGFß type II receptor protein expression levels were slightly increased during the first 3 days; the RNA and protein expression levels of other growth factors and receptors did not change in submucosal or muscle cells during the regeneration process.

Table 1. Changes in morphology and proliferation of the regenerating urothelium.

time after wounding (h)	control	1	4	8	16	24	72	110	240
L.I.	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	1.9 ± 1.9	31.2 ± 9.3	21.9 ± 8.5	3.5 ± 1.3	2.1 ± 2.1	0.2 ± 0.2
cell layers	3	0-3	0-3	0-3	0-3	1-3	3-6	3-6	3-4
terminal differentiation	+	—	—	—	—	—	—/+	—/+	+

Table 1. Morphological and immunohistochemical analysis of regenerating bladder urothelium. The proliferation is given as the mean labelling index of urothelia from 6 bladders ± the standard deviation (L.I., see materials and methods section). The urothelial maturation and differentiation status is given by the minimal and maximal number of mucosal cell layers in 6 bladders (cell layers), and the presence of superficial cells with the typical features of umbrella cells within the regenerating area (terminal differentiation). + = umbrella cells present; — = umbrella cells absent; —/+ = superficial cells different from intermediate cells.

DISCUSSION

Here we described several aspects of urothelial regeneration in vivo: differentiation and maturation by morphological analysis, proliferation by immunohistochemistry, and localization of several growth factors and their

receptors by in situ hybridization and immunocytochemistry. We demonstrated that re-epithelialization of mouse urothelium in vivo started within 4 hours after the damage, and that this re-epithelialization by one cohesive sheet of flattened cells was completed within approximately 24 hours. This early onset and rapid regeneration has also been described for damaged gastric mucosa in rats after exposure to ethanol (6). During the early phase of regeneration, urothelial cells lining the wound, and adjacent to intact mucosa, were flattened and elongated representing the features of migrating cells. Proliferation activity was particularly enhanced near the wound edges of eroded areas. These observations support the idea that urothelial re-epithelialization is brought about by both an enhanced migration and proliferation analogous to skin wound repair (33). The second phase in urothelial regeneration seems to be reflected by the stratification into multiple cell layers resulting in hyperplasia at day 3 to 5. In the third phase the hyperplastic urothelium returns to the original state of a structurally normal urothelium within 5 to 10 days after the damage.

In vivo studies located the expression of several of these growth factors and/or their receptors within the epidermis during the process of skin wound healing (7,11,14,34). Several studies demonstrated that exogenous application of epidermal growth factor (EGF), transforming growth factor ß (TGFß), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) to skin wounds enhanced epithelial regeneration in vivo (9,10,13). In vitro studies pointed out that these growth factors directly affected proliferation, differentiation, migration, and synthesis of extracellular matrix not only in mesenchymal cells, but also in keratinocytes (35-39). These data suggest that growth factors can regulate the epithelial regeneration not only by paracrine stroma-epithelium interactions, but also by direct interaction of growth factors with their cognate receptors within the epidermis.

In the present model for acute urothelial injury, the lesions were predominantly confined to the mucosa, while inflammatory infiltrates were absent. Except for $TGF\beta_1$ and TGFß type II receptor, changes in RNA or protein expression of growth factors or receptors were exclusively detected in the urothelium. This implies that the urothelial regeneration in this model does not require a paracrine action of stroma-derived growth factors. Such an autonomous stroma-independent mechanism for epithelial regeneration has also been suggested for acute injured skin epithelium during which both growth factors and their receptors were expressed within the epithelial cells (11,12,34).

High levels of $TGF\beta_1$ RNA were found during the re-epithelialization and

the differentiation phases of wound healing, both in regenerating skin epithelium (40,41) and in regenerating bladder epithelium (this study). This phenomenon agrees with the biological effects of these growth factors on epithelial cells in vitro. In vitro studies showed that TGFß$_1$ indeed induced differentiation of keratinocytes, bronchiocytes, and urothelial cells in primary culture (21,38,42). On the other hand, both TGFß$_1$ and the TGFß type II receptor were expressed in vivo in migrating keratinocytes as well as urothelial cells adjacent to the site of injury (34,40; this study). This is in concordance with in vitro studies, which demonstrated that TGFß stimulated the migration of undifferentiated keratinocytes only (8,35). As with IGFs and FGFs, the TGFß-dependent epithelial migration process is probably mediated by growth factor-induced synthesis and secretion of the extracellular matrix proteins fibronectin and collagen type I and type IV in both mesenchymal and epithelial cells (35,43), and a concomittant expression of specific receptors for fibronectin, α5ß1 integrin, and for collagen, α2ß1 integrin (43,44).

The mucosal RNA expression of FGF-1, but not FGF-2, coincided with the migration and the proliferation phases during urothelial regeneration. A similar spatio-temporal expression pattern was seen during epidermal regeneration (12). Previous studies demonstrated that FGF-1 could induce the proliferation of several murine urothelial cell lines (18,20) and that FGF-1, but not FGF-2, stimulated the migration of a murine urothelial cell line (19). In addition, studies on primary cultures of urothelial cells pointed out that FGF-1, but not FGF-2, could induce proliferation and migration of these cells, but not differentiation (21). These in vitro data are in line with the present in vivo observation, suggesting that FGF-1 is a more relevant physiological factor than FGF-2 in stimulating migration and proliferation of urothelial cells in vivo. In vitro studies on keratinocytes showed that FGF-1 could induce both their proliferation and migration (37,39,45). Furthermore, Hannson and Norstrom (15) demonstrated an enhanced expression of FGF-1 in regenerating stomach epithelium in vivo. Apparently, the direct interaction of FGFs with epithelial cells to stimulate migration and proliferation represents a common mechanism in a variety of epithelia. Expression of other growth factors, like FGF-4 which affects urothelial cells in vitro (46), and FGF-7 (14), were not investigated by us but may also be important for the wound healing process in the bladder.

Whereas the elevation in IGF-II RNA expression was limited, type I IGF receptor RNA expression in the urothelium was strongly enhanced within 24 hours after injury. In contrast, Antoniades et al (12) failed to observe such a

change in IGF or IGF receptor expression in skin epithelium during regeneration. Specific IGFBPs have been identified that can inhibit IGF-mediated effects by preventing the IGFs to bind to their receptor (22). Albiston et al (47) noted that during regeneration of damaged ileum epithelium, the RNA level of IGF-I did not change, while IGFBP-3 RNA levels decreased, suggesting a net increase in the availability of IGF-I for its receptor. Other studies demonstrated an enhanced IGF-I RNA and protein expression in regenerating, undifferentiated epithelial cells of the rat ear and the rat kidney (7,48). In our model for acute urothelial damage, IGFBP-2 RNA was expressed constitutively and predominantly in basal urothelial cells. This expression did not change during regeneration. Thus, in our model the alterations in type I IGF receptor rather than IGF or IGFBP expression seem to play a regulatory role. The in vivo function of IGFs during epithelial regeneration is not clear yet. Both IGF-I and IGF-II can act as progression factors in the cell cycle (49). IGF-I also induces the proliferation, migration and differentiation of keratinocytes in vitro (36,50). Both the expression of IGF-II and the enhanced type I IGF receptor RNA expression in regenerating urothelium in particular during the migration and proliferation phases may be in line with an autocrine effect of IGF-II on urothelial migration and the progression through the cell cycle needed for proliferation of urothelial cells. Moreover, the growth promoting actions of IGF-II may be potentiated by the urothelium-derived IGFBP-2 (22)

A high constitutive expression of PDGF-A RNA was observed in mouse urothelium, while a small increase in PDGF-B RNA was noted after injury. Although we detected a low PDGF ß receptor RNA expression in mouse urothelium, this was only noted during one hybridization. PDGF α receptor RNA was not detected. Hence, we are now unable to predict any function of PDGFs during wound healing in the bladder in vivo. A role for PDGF in epidermal regeneration has been suggested by several studies (9-11).

Antoniades et al (12) showed that both epidermal TGFα and EGF receptor RNA expression were enhanced after skin damage, indicating an autocrine role for TGFα in epidermal repair. Although EGF in the serous salivary glands of mice was stained with a specific antibody against mouse EGF, we could not detect EGF expression in the bladder (data not shown). However, it cannot be excluded that EGF/TGFα are also involved in urothelial regeneration analogous to skin epithelium. Indirect evidence for a paracrine role for EGF is provided by the observation that EGF has been shown to enhance proliferation and migration of mouse urothelial cells in primary culture (21), and that EGF stimulates the

uptake of tritiated thymidine by basal rat urothelial cells in vivo (17).

In conclusion, in this model for regeneration of acute injured urothelium the alterations in growth factor and receptor expression (RNA and protein) were mainly confined to the urothelium, indicating that urothelial repair is predominantly driven by the urothelium itself. The growth factor and receptor expression in regenerating transitional epithelium correlated with specific phases in the repair process and with the effects of these growth factors on urothelial cells in primary culture. Our data show a great similarity with expression patterns of growth factors and receptors during epidermal wound healing in vivo, suggesting a more general mechanism for epithelial regeneration. The present data may also provide a basis to explain biological consequences of an aberrational growth factor or its receptor expression in transitional cell carcinomas.

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LITERATURE

1 Hicks RM: The mammalian bladder: an accommodating organ. Biol Rev 1975, 50:215-246.

2 Cohen SM: Toxic and nontoxic changes induced in the urothelium by xenobiotics. Toxicol Appl Pharmacol 1989, 101:484-498.

3 Tong Y-C, Monson FC, Erika B, Levin RM: Effects of acute in vitro overdistension of the rabbit urinary bladder on DNA synthesis. J Urol 1992, 148:1347-1350.

4 Post JG, te Poele JAM, Oussoren Y, Stewart FA: Bladder damage in mice after single and repeated intravesical instillations of mitomycin C or doxorubicin. J Urol 1993, 150:1965-1969.

5 Hudspeth AJ: Establishment of tight junctions between epithelial cells. Proc Natl Acad Sci USA 1975, 72:2711-2713.

6 Silen W, Ito S: Mechanisms for rapid re-epithelialization of the gastric mucosal surface. Ann Rev Physiol 1985, 47:217-229.

7 Jennische E, Skottner A, Hansson H-A: Dynamic changes in insulin-like growth facor I immunoreactivity correlate to repair events in rat ear after freeze-thaw injury. Exp Mol Pathol 1987, 47:193-201.

8 Hebda PA: Stimulatory effects of transforming growth factor-beta and epidermal growth factor on epidermal cell outgrowth from porcine skin explant cultures. J Invest Dermatol 1988, 91:440-445.

9 Lynch SE, Colvin RB, Antoniades HN: Growth factors in wound healing. Single and synergistic effects on partial thickness porcine skin wounds. J Clin Invest 1989, 84:640-646.

10 Mustoe TA, Pierce GF, Morishima C, Deuel TF: Growth factor-induced accelaeration of tissue repair through direct and inductive activities in a rabbit dermal ulcer model. J Clin Invest 1991, 87:694-703.

11 Antoniades HN, Galanopoulos T, Neville-Golden J, Kiritsy CP, Lynch SE. Injury induces *in vivo* expression of platelet-derived growth factor (PDGF) and PDGF receptor mRNAs in skin epithelial cells and PDGF mRNA in connective tissue fibroblasts. Proc Natl Acad Sci USA 1991, 88:565-569.

12 Antoniades HN, Galanopoulos T, Neville-Golden J, Kiritsy CP, Lynch SE: Expression of growth factor and receptor mRNAs in skin epithelial cells following acute cutaneous injury. Am J Pathol 1993, 142:1099-1110.

13 Mellin TN, Mennie RJ, Cashen DE, Ronan JJ, Capparella J, James ML, Disalvo J, Frank J, Linemeyer D, Giminez-Gallego G, Thomas KA: Acidic fibroblast growth factor accelerates dermal wound healing. Growth Factors 1992, 7:1-14.

14 Werner S, Peters KG, Longaker MT, Fuller-Pace F, Banda MJ, Williams LT: Large induction of keratinocyte growth factor expression in the dermis during wound healing. Proc Natl Acad Sci 1992, 89:6896-6900.

15 Hannson HA, Norstrom E: aFGF, EGF and their receptors in the normal and healing rat gastric mucosa. Digestion 1991, 49 Suppl:17-18.

16 Fitzpatrick LR, Jakubowska A, Martin GE, Davis M, Jaye MC, Dionne CA: Acidic fibroblast growth factor accelerates the healing of acetic-acid-induced gastric ulcers in rats. Digestion 1992, 53:17-27.

17 Messing EM, Hanson P, Ulrich P, Erturk E: Epidermal growth factor interactions with normal and malignant urothelium: *in vivo* and *in situ* studies. J Urol 1987, 138:1329-1335.

18 Vallés AM, Boyer B, Badet J, Tucker GC, Barritault D, Thiery JP: Acidic fibroblast growth factor is a modulator of epithelial plasticity in a rat bladder carcinoma cell line. Proc Natl Acad Sci USA 1990, 87:1124-1128.

19 Jouanneau J, Gavrilovic J, Caruelle D, Jaye M, Moens G, Caruelle J-P, Thiery J-P: Secreted or nonsecreted forms of acidic fibroblast growth factor produced by transfected epithelial cells influence cell morphology, motility, and invasive potential.

Proc Natl Acad Sci USA 1991, 88:2893-2897.

20 De Boer WI, Rebel JMJ, Foekens JA, Vermey M, Van der Kwast TH: Characterization of mouse urothelial cell lines in different phases of transitional-cell carcinogenesis. Int J Cancer 1993, 54:1022-1027.

21 De Boer WI, Rebel JMJ, Vermey M, De Jong AAW, Van der Kwast TH: Characterization of distinct functons for growth factors in murine transitional epithelial cells in primary organotypic culture. Exp Cell Res 1994, 214:510-518.

22 Clemmons DR: IGF binding proteins: regulation of cellular action: Growth Regulation 1992, 2:80-87.

23 Schuller AGP, Lindenbergh-Kortleve DJ, De Boer WI, Zwarthoff EC, Drop SLS: Localization of the epitope of a monoclonal antibody against human insulin-like growth factor binding protein-1, functionally interfering with insulin-like growth factor binding. Growth Regulation 1993, 3:32-34.

24 Lee K-H, Bowen-Pope D, Reed RR: Isolation and characterization of the *α* platelet-derived growth factor receptor from rat olfactory epithelium. Mol Cell Biol 1990, 10:2237-2246.

25 Brewitt B, Clark JI: Growth and transparency in the lens, an epithelial tissue, stimulated by pulses of PDGF. Science 1988, 242:777-779.

26 Campochiaro PA, Glaser BM: Platelet-derived growth factor is chemotactic for human retinal pigment epithelial cells. Arch Ophthalmol 1985, 103:576-579.

27 Kimball ES, Bohn WH, Cockley KD, Warren TC, Sherwin SA: Distinct high-performance liquid chromatography pattern of transforming growth factor activity in urine of cancer patients with that of normal individuals. Cancer Res 1984, 44:3616-3619.

28 Carpenter G: Epidermal growth factor: biology and receptor metabolism. J Cell Sci Suppl 1985, 3:1-9.

29 Kawamata H, Azuma M, Kameyama S, Nan L, Oyasu R: Effect of epidermal growth factor/transforming growth factor alpha and transforming growth factor beta 1 on growth in vitro of rat urinary bladder carcinoma cells. Cell Growth Differ 1992, 3:819-825.

30 Coombs LM, Pigott DA, Eydmann ME, Proctor AJ, Knowles MA: Reduced expression of TGF beta is associated with advanced disease in transitionalcell carcinoma. Br J Cancer 1993, 67:578-584.

31 Schuller AGP, Zwarthoff EC, Drop SLS: Gene expression of the six insulin-like growth factor binding proteins in the mouse conceptus during mid-

and late gestation. Endocrinology 1993, 132:2544-2550.

32 Schutte B, Reynders MMJ, Van Assche CLMVJ, Hupperets PSGJ, Bosman FT, Blijham GH: Studies with anti-bromodeoxyuridine antibodies. Cytometry 1987, 8:372-376.

33 Odland G, Ross R: Human wound repair. I. Epidermal regeneration. J Cell Biol 1968, 39:135-151.

34 Schmid P, Cox D, Bilbe G, McMaster G, Morrison C, Stähelin H, Lüscher N, Seiler W: TGF-ßs and TGF-ß type II receptor in human epidermis: differential expression in acute and chronic skin wounds. J Pathol 1993, 171:191-197.

35 Nickoloff BJ, Mitra RS, Riser BL, Dixit VM, Varani J: Modulation of keratinocyte motility. Correlation with production of extracellular matrix molecules in response to growth promoting and antiproliferative factors. Am J Pathol 1988, 132:543-551.

36 Ristow HJ, Messmer TO: Basic fibroblast growth factor and insulin-like growth factor I are strong mitogens for cultured mouse keratinocytes. J Cell Physiol 1988, 137:277-284.

37 Shipley GD, Keeble WW, Hendrickson JE, Coffey RJ, Pittelkow MR: Growth of normal human keratinocytes and fibroblasts in serum-free medium is stimulated by acidic and basic fibroblast growth factor. J Cell Physiol 1989, 138:511-518.

38 Glick AB, Danielpour D, Morgan D, Sporn MB, Yuspa SH: Induction and autocrine receptor binding of transforming growth factor-ß2 during terminal differentiation of mouse keratinocytes. Mol Endocrinol 1990, 4:46-52.

39 Tsuboi R, Sato C, Shi CM, Ogawa H: Stimulation of keratinocyte migration by growth factors. J Dermatol 1992, 19:652-653.

40 Kane CJM, Hebda PA, Mansbridge JM, Hanawalt PC: Direct evidence for spatial and temporal regulation of transforming growth factor ß1 expression during cutaneous wound healing. J Cell Physiol 1991, 148:157-173.

41 Levine JH, Moses HL, Gold LI, Nanney LB: Spatial and temporal patterns of immunoreactive transforming growth factor beta 1, beta 2, and beta 3 during excisional wound repair. Am J Pathol 1993, 143:368-380.

42 Sacco O, Romberger D, Rizzino A, Beckmann JD, Rennard SI, Spurzem JR: Spontaneous production of transforming growth factor-ß2 by primary cultures of bronchial epithelial cells. J Clin Invest 1992, 90:1379-1385.

43 Kagami S, Border WA, Ruoslahti E, Noble NA: Coordinated expression of beta 1 integrins and transforming growth factor-beta-induced matrix proteins in

glomerulonephritis. Lab Invest 1993, 69:68-76.

44 Scharfetter-Kochanek K, Klein CE, Heinen G, Mauch C, Schaefer T, Adelmann-Grill BC, Goerz G, Fusenig NE, Krieg TM, Plewig G: Migration of a human keratinocyte cell line (HACAT) to interstitial collagen type I is mediated by the $\alpha 2\beta 1$-integrin receptor. J Invest Dermatol 1992, 98:3-11.

45 Gospodarowicz D, Plouet J, Malerstein B: Comparison of the ability of basic and acidic fibroblast growth factor to stimulate the proliferation of an established keratinocyte cell line: modulation of their biological effects by heparin, transforming growth factor beta (TGF beta), and epidermal growth factor (EGF). J Cell Physiol 1990, 142:325-333.

46 Tucker GC, Delouvée A, Jouanneau J, Gavrilovic J, Moens G, Vallés AM, Thiery JP: Amplification of invasiveness in organotypic cultures after NBT-II rat bladder carcinoma stimulation with in vitro scattering factors. Invasion Metastasis 1991, 11:297-309.

47 Albiston AL, Taylor RG, Herington AC, Beveridge DJ, Fuller PJ: Divergent ileal IGF-I and IGFBP-3 gene expression after small bowel resection: a novel mechanism to amplify IGF action? Mol Cell Endocrinol 1992, 83:R17-R20.

48 Matejka GL, Jennische E: IGF-I binding and IGF-I mRNA expression in the post-ischemic regenerating rat kidney. Kidney Int 1992, 42:1113-1123.

49 Aaronson SA: Growth factors and cancer. Science 1991, 254:1146-1153.

50 Kamalati T, Howard M, Brooks RF: IGF I induces differentiation in a transformed human keratinocyte line. Development 1989, 106:283-293.

Chapter 5.

CHARACTERIZATION OF MOUSE UROTHELIAL CELL LINES IN DIFFERENT PHASES OF TRANSTIONAL CELL CARCINOGENESIS.

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CHARACTERIZATION OF MOUSE UROTHELIAL CELL LINES IN DIFFERENT PHASES OF TRANSITIONAL-CELL CARCINOGENESIS

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Altered cellular responsiveness to growth factors is one of the factors involved in carcinogenesis. In order to study the role of growth factors in transitional-cell carcinogenesis, we established 3 urothelial cell lines from normal mouse urothelium, designated g/G, NUC-5 and NUC-1. These cell lines were studied by light and transmission electron microscopy, karyotyping, grafting in syngeneic mice, growth-factor response *in vitro* under serum-free conditions, and EGF receptor expression. In the presence of insulin or insulin-like growth factors I and II, proliferation of the non-tumorigenic DNA-tetraploid g/G and DNA aneuploid NUC-5 cells is stimulated by EGF, TGFα, bFGF and aFGF. This stimulation can be abolished in g/G but not in NUC-5 cells by simultaneous addition of TGFβ. Proliferation of g/G and NUC-5 cells can also be stimulated by PDGF-AA. The spindle-cell-like NUC-1 cells are DNA aneuploid and tumorigenic in syngeneic mice; they express low levels of EGF receptors and their autonomous proliferation is only affected by insulin or insulin-like growth factors. Each of these cell lines seems to reflect a different phase in transitional-cell carcinogenesis: g/G cells have gained immortality, have become tetraploid, but are non-tumorigenic and growth-factor-dependent. NUC-5 cells have become aneuploid, have a growth-factor responsiveness different from that of normal epithelial cells, but are still non-tumorigenic. NUC-1 cells are aneuploid, tumorigenic, and growth-factor-independent. These urothelial cell lines provide a suitable tool for further studies in transitional-cell carcinogenesis.

It has become well established that growth factors are involved in pathophysiological processes such as wound healing (Mustoe *et al.*, 1991), cancer development (Aaronson, 1991), tumour-cell migration and invasion (Jouanneau *et al.*, 1991). Also, in the development and progression of transitional-cell carcinoma (TCC) of the bladder, a malignancy with a high recurrence rate (Raghavan *et al.*, 1990), growth factors may be involved. While normal urine only contains EGF in high concentrations (Hirata and Orth, 1979; Carpenter, 1985), urine of patients with bladder cancer contains both TGFα and bFGF (Kimball *et al.*, 1984; Chodak *et al.*, 1988). Chodak *et al.* (1986) found that mouse bladder tumours contain an FGF-like factor.

The presence of bFGF might result in a selective growth advantage of urothelial tumour cells over their normal counterparts, while EGF-like growth factors might be involved in the induction of characteristics of neoplastic cells (Knowles *et al.*, 1985; Yura *et al.*, 1989). Several *in vitro* studies have documented the effects of growth factors on proliferation of urothelial cells (Knowles *et al.*, 1985; Messing *et al.*, 1987; Messing and Reznikoff, 1987; Dubeau and Jones, 1987). However, in those studies the influence of only a limited number of growth factors on primary urothelium or on a single cell line was examined.

Our aim was to investigate *in vitro* which growth factors are involved in regulation of proliferation of urothelial cells, and to correlate this response with the characteristics of transformation. To this end, we developed 3 mouse urothelial cell lines through long-term propagation of mouse urothelial explant cultures. We characterized these cell lines with respect to morphology, DNA ploidy, and tumorigenicity. Proliferation of the cell lines was determined after treatment with various growth factors, known to affect proliferation of other epithelial cell types. This was done in serum-free medium to exclude possible interference with other factors contained in serum. The results suggest that each cell line represents a different phase in transitional-cell carcinogenesis.

MATERIAL AND METHODS

Chemicals

Culture media and supplements were purchased from Flow (Zwanenburg, NL) and Sigma (St. Louis, MO), respectively. Mouse EGF and human TGFβ$_1$ were obtained from Sigma, human bFGF and human TGFα from Oncogene Science (Uniondale, NY), and human IGF-I, IGF-II, aFGF, and PDGF-AA from Boehringer Mannheim (Almere, NL). Porcine heparin was purchased from UBI (Lake Placid, NY). Tritiated thymidine was purchased from Amersham ('sHertogenbosch, NL).

Animals

Female C3H/Law and C3H/El mice, as well as nude BALB/c mice held in macrolon cages, were fed *ad libitum* with chow from Hope Farms (Woerden, NL). Urothelial bladder explants from neonatal mice (3–5 days) and subsequent urothelial cell cultures were obtained and maintained as described (Van der Kwast *et al.*, 1989).

Cell culture

Routine medium consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F10, supplemented with 10% foetal calf serum (FCS; heat-inactivated), 5 μg/ml insulin (unless stated otherwise), 5 μg/ml transferrin, 50 nM hydrocortisone, and 5 ng/ml selenite. Furthermore, 100 IU penicillin/ml and 100 μg/ml streptomycin were added. The serum-free medium was similar to the routine culture medium but without the FCS, and supplemented with 0.1% bovine serum albumin (BSA), 4 μM spermine, 4 μM spermidine, 0.1 mM ethanolamine and 1 μM putrescine. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2, in 167-cm^2 culture flasks (Costar, Cambridge, MA) in routine medium. Passage of cells was done by trypsinization. Experiments were performed in serum-free medium. After incubation for 45 min with serum-free medium and rinsing with PBS, pH 7.2, cells were trypsinized and viable cells were counted. Next, cells were plated in serum-free medium at non-confluence (*i.e.*, 10,000 cells per well) in 96-multi-well dishes (NUNC, Roskilde, Denmark). Cells were cultured for 96 hr. After the first 20 hr, growth factors or FCS were added in concentrations ranging from 0.1 ng/ml to 50 ng/ml to a final volume of 200 μl per well.

Extracellular matrix coating

In experiments using extracellular matrix (ECM) coatings, wells were pre-coated for 1 hr with 25 μg/ml fibronectin (Sigma) or for 2 hr with 25 μg/ml human collagen type IV (kindly donated by Dr. J.P.M. Cleutjens, Dept. of Pathology,

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University of Limburg, NL) prior to cell plating. This concentration yielded optimal cell adhesion for both matrix components. The remaining coating was aspirated before plating of the cells. When cultured on BSA in the absence of FCS, both g/G and NUC-5 cells adhered to the culture substratum, but NUC-1 cells did not. Tritiated thymidine incorporation into cells cultured on BSA was approximately equal (g/G; NUC-5) or at least 20 times reduced (NUC-1) as compared to cells cultured on fibronectin (De Boer *et al.*, 1992). Based on the above data, the present experiments on growth-factor responsiveness of the 3 cell lines were performed on fibronectin-coated wells.

^{3}H-thymidine incorporation

During the final 16 hr of culture, cells were incubated with 0.5 μCi ^{3}H-thymidine/well, then cells were trypsinized and harvested. The incorporated radioactivity was counted using a BetaPlate scintillation counter (LKB-Pharmacia, Woerden, NL), and expressed as counts per minute (cpm).

Transmission electron microscopy

For transmission electron microscopy, cells were cultured on Cyclopore membranes (pore size 0.45 μm) (Falcon, Becton-Dickinson, Etten-Leur, NL) under routine culture conditions. Post-confluent cultures were rinsed with PBS, pH 7.2, and fixed with 1.5% w/v glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. After rinsing with cacodylate buffer pH 7.4, cells were post-fixed with 1% w/v osmium tetroxide and 1.5% w/v potassium ferrocyanide in cacodylate buffer. After dehydration, cells were embedded in epon (Zeiss, Weesp, NL). Ultra-thin sections were contrasted on 200-mesh copper grids with uranyl acetate and lead-nitrate. Sections were studied using a transmission electron microscope (Zeiss EM902) at 80 kV.

Cytokeratin expression

Cells were cultured in routine culture medium. Incubations were aborted by rinsing with PBS, pH 7.2, and fixed with ethanol. Cytokeratin expression was subsequently determined using a polyclonal antibody (Dakopatts, ITK Diagnostics, Uithoorn, NL) directed against multiple cytokeratins. This antibody reacted with normal mouse urothelium.

EGF receptor expression

Cells were cultured to subconfluence in 167-cm^2 flasks under routine culture conditions. Then cells were rinsed with PBS, incubated for another hour in serum-free medium without insulin, hydrocortisone, or transferrin, and again rinsed with PBS before harvesting by scraping in PBS. Cells were homogenized and EGF receptor expression was determined in the resulting membrane fractions by incubation for 20 hr at 20°C with increasing concentrations (0.1 to 2 nM) of ^{125}I-EGF exactly as described by Foekens *et al.* (1992). Mouse receptor grade EGF (Sigma) was radiolabelled to ^{125}I-EGF (specific activity 466 Ci/mmol) with Protag 125 (J.T. Baker, Philipsburg, NJ) as described by Benraad and Foekens (1990). Receptor expression in 3 samples was calculated by Scatchard analysis.

DNA-ploidy determination

Cells growing logarithmically, in routine medium, were arrested in metaphase by 0.1 μg/ml colchicine and chromosome analysis was performed by direct counting of chromosomes (Priest, 1969). To establish the number of chromosomes a total of 50 cells in metaphase per cell line was counted.

RESULTS

NUC-1 and NUC-5 cells were derived from different C3H/Law mice, while g/G cells were derived from a C3H/El mouse. Characteristics of the 3 cell lines are presented in Table I. Characterization of the cell lines was done in passages 18 to 28. The cell lines have now been propagated for at least 40 passages while maintaining their morphological characteristics.

Morphology and DNA-ploidy

The morphological differences between the cell lines are shown in Figure 1, by both light and transmission electron microscopy. The g/G cells are cobblestone-like (Fig. 1*a*), while NUC-5 cells are large cells with long cellular extrusions and no clear cell boundaries, indicating flattening (Fig. 1*c*). Both g/G and NUC-5 cells show stratification up to a maximum of 3 cell layers (Fig. 1*b, d*). NUC-1 cells have a spindle-cell-like morphology, grow in high cell densities arranged in a disorderly manner in several cell layers, and contain several nucleoli per nucleus (Fig. 1*e*). Cells of all 3 lines have many lysosomes and autophagic bodies, and active endocytosis, microvilli on the superficial cells, and desmosomes (Fig. 1*b, d, f*). Frequently, interdigitations can be observed in the lateral cellular membranes of adjacent cells. Immunocytochemistry revealed, in all 3 cell lines, the presence of cytokeratin. Analysis of metaphase chromosome numbers shows that g/G passage-28 cells are DNA tetraploid, while NUC-1 passage-24 and NUC-5 passage-18 cells are DNA aneuploid.

Tumorigenicity

No tumours arose within 2 months upon s.c. inoculation of 5×10^6 g/G or NUC-5 cells, either in syngeneic C3H mice or in nude mice. Inoculation of 5×10^6 cells NUC-1 resulted in

TABLE I – CHARACTERIZATION OF CELL LINES *IN VITRO*

Cell line	g/G	NUC-5	NUC-1
Morphology			
Cells	Cobblestone-like	Large, flat	Spindle-cell-like
Arrangement in layers	Regular	Regular	Disordered
Number of cell layers	≤3 cell layers	≤3 cell layers	Multilayered
Junctional complexes/desmosomes	+[1]	+	+
Microvilli	+	+	+ (few)
Autophagic bodies	+	+	+
Cytokeratin	+	+	+
Chromosome number	80	70–75	70–75
Tumorigenic	No	No	Yes; invasive
EGF receptor expression[2]			
Binding (fmol/mg)	123.4 ± 12.5	50.9 ± 1.1	2.6
K_D (pM)	228.6 ± 25.8	277.2	197.2
Protein (pg/cell)	336 ± 39	409 ± 47	253 ± 22

[1]+, Present.–[2]Binding capacity is expressed as fmol ^{125}I-EGF per mg membrane protein; ^{125}I-EGF binding affinity is expressed as pM; protein content per cell is expressed in pg.

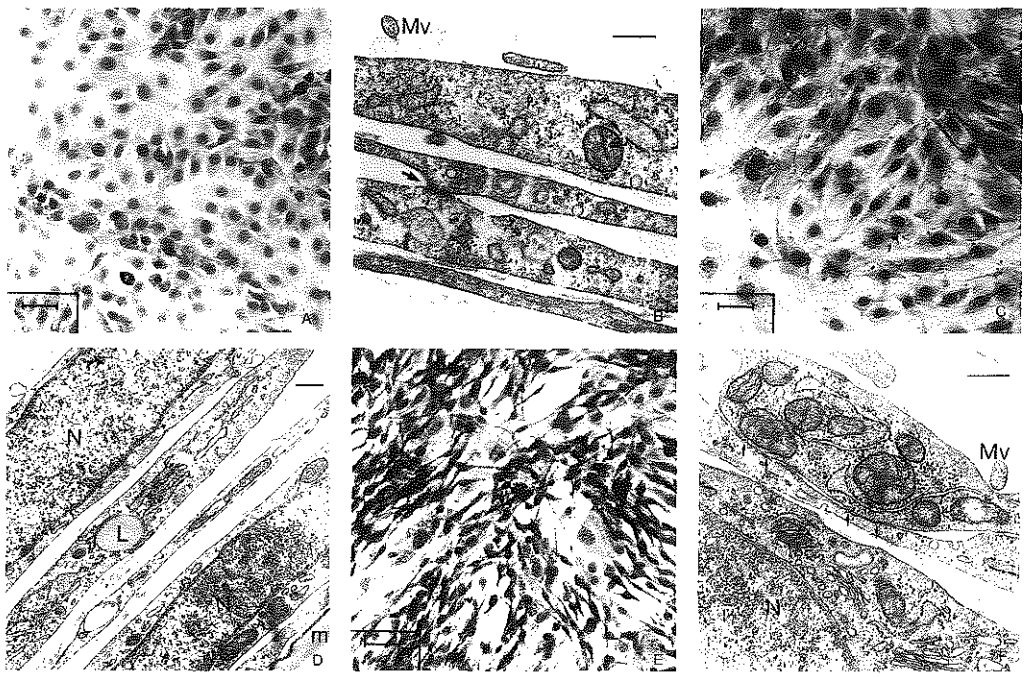

FIGURE 1 – Morphology of g/G *(a, b)*, NUC-5 *(c, d)*, and NUC-1 *(e, f)*. *a, c* and *e* are light microscopic photographs of Giemsa-stained cultures; bar = 20 μm. *b, d* and *f* are transmission electron microscopic photographs; bar = 0.4 μm. L = lipid droplet; Mv = microvillus; N = nucleus; arrow = desmosome; m = membrane.

tumours in 10 out of 10 syngeneic C3H mice after 2 weeks, necessitating the termination of the experiment within 1 month. These tumours exhibited invasive growth into s.c. muscle layers. No lymphogenic or haematogenic metastases were observed. I.V. injection of NUC-1 cells in the tail vein led to colonization of the lungs.

Growth-factor response

Figure 2 summarizes the results of ^{3}H-thymidine incorporation in all 3 cell lines cultured on fibronectin under serum-free conditions, with or without the indicated growth factors. ^{3}H-thymidine incorporation in g/G and NUC-5 cells was modulated by insulin, IGF-I, and IGF-II only in the presence of another growth factor. In the presence of insulin, aFGF in the presence of 1.8 units/ml heparin, bFGF, and EGF-like growth factors induced proliferation of g/G and NUC-5 cells dose-dependently, which could be inhibited by TGFβ in g/G cells. In contrast to g/G cells, TGFβ induced proliferation of NUC-5 cells with an ED_{50} of 0.6 ng/ml, reaching maximal stimulation at 1 ng/ml (Fig. 2*d*). Moreover, 1 ng/ml of TGFβ enhanced the EGF-, TGFα- or bFGF-induced proliferation of NUC-5 cells synergistically.

Since our 3 cell lines expressed PDGF type-α receptor RNA but not PDGF type-β receptor RNA (data not shown) we analyzed whether they expressed functional PDGF type-α receptors. Therefore, we tested the proliferation-stimulating effect of PDGF-AA which only binds to the type-α receptor, on our cell lines. PDGF-AA increased proliferation of g/G and NUC-5 proliferation maximally at 20 ng/ml 2.5-fold (Fig. 2).

In contrast to g/G and NUC-5 cells, NUC-1 cells proliferate autonomously. Incorporation of ^{3}H-thymidine in NUC-1 cells was only affected by IGF-I, insulin, or, although less effectively, IGF-II (Fig. 2).

EGF-receptor expression

Finally, we determined EGF receptor (EGF-R) expression in subconfluent cell cultures, maintained in routine culture medium. Table I shows one high-affinity class of EGF-R in all 3 cell lines (K_D = 200–300 pM). The EGF binding capacities differed from one cell line to another. Notably, NUC-1 cells were least affected by EGF-like growth factors and expressed the lowest EGF binding capacity. The opposite holds true for g/G cells.

DISCUSSION

In this study we characterized 3 spontaneously immortalized mouse urothelial cell lines. Such spontaneous immortalization occurs frequently after long-term *in vitro* propagation of urothelial cells derived from mouse bladder explant cultures (Franks and Henzell, 1970; Van der Kwast *et al.*, 1989). Different parameters were examined: morphology by light and transmission electron microscopy, tumorigenicity, ploidy status, response to growth factors, and EGF-R expression. All 3 cell lines have characteristics of epithelial cells. Based upon their characteristics, we propose that g/G, NUC-5 and NUC-1 cells reflect distinct phases in urothelial-cell carcinogenesis. This proposal is based upon the currently accepted concepts regarding cell morphology, genetic alterations, growth-factor response, and growth-factor receptor expression during carcinogenesis.

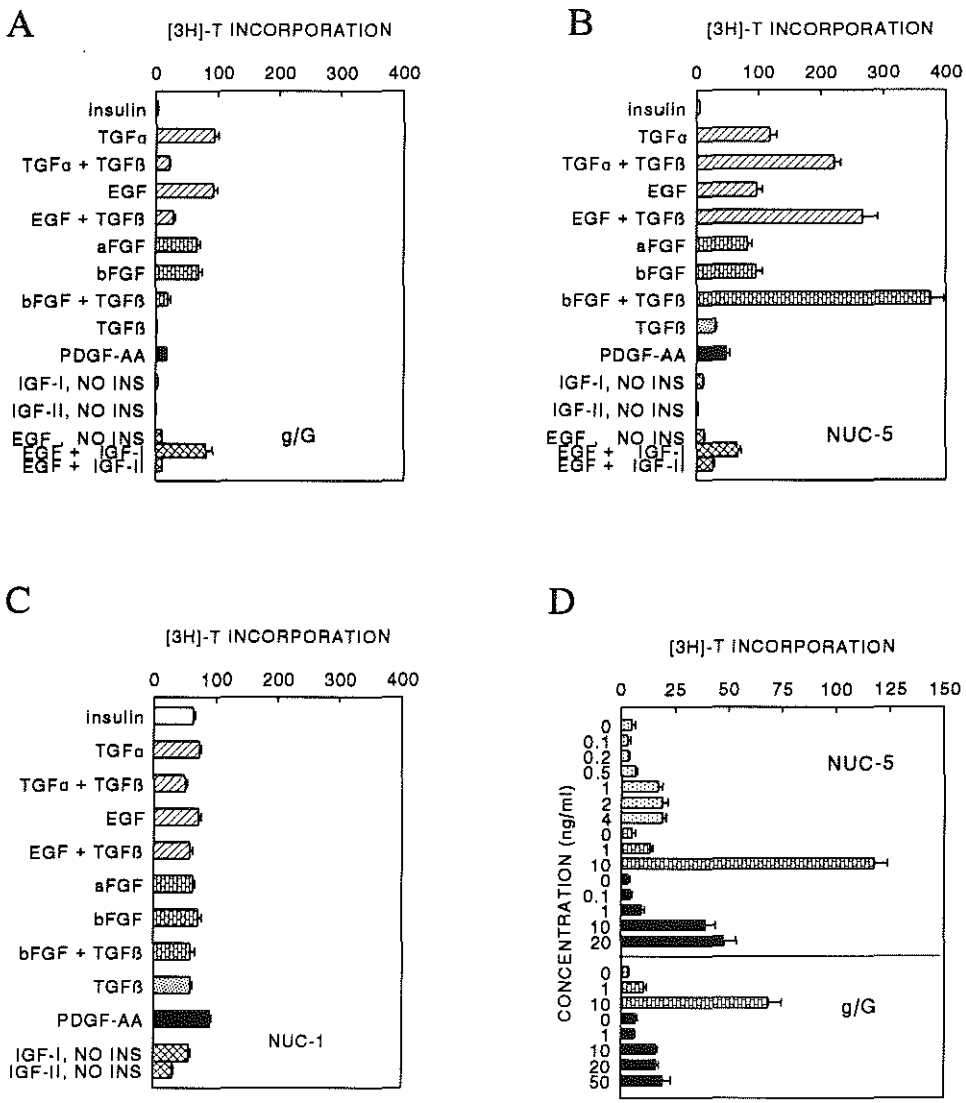

FIGURE 2 – ^{3}H-thymidine incorporation into g/G *(a)*, NUC-5 *(b)*, or NUC-1 *(c)* after 72 hr of incubation in serum-free medium with the indicated growth factors. Maximal ^{3}H-thymidine incorporations are measured in the presence of 2.5% FCS and are 3.3×10^4 cpm (g/G), 2.8×10^4 cpm (NUC-5), and 2.6×10^5 cpm (NUC-1). These values were taken as 100%. Results in *a–c* are shown for the following concentrations of the growth factors: EGF, TGFα and TGFβ, 1 ng/ml; aFGF, bFGF, IGF-I and IGF-II, 10 ng/ml; PDGF-AA, 20 ng/ml. Studies with IGF-I or IGF-II were performed in the absence of insulin. Figure 2*d* shows a dose-dependent response to TGFβ (stippled bars) for NUC-5 cells, to bFGF (squared bars) and PDGF-AA (closed bars) for both g/G and NUC-5 cells. The concentration of growth factors is given on the left axis, the proliferation response on the upper axis. Bars represent data from 8 wells ± S.E.M. T = thymidine; INS = insulin.

Morphology

The g/G cells most closely resemble the normal transitional epithelium (regularly arranged, cobblestone-like cells). They share features of basal or intermediate urothelial cells in having many lysosomes, autophagic bodies and free ribosomes (Hicks, 1975). NUC-5 cells seem to resemble differentiated transformed cells in being large and flattened, with many lateral membrane interdigitations, suggesting an intermediate or superficial urothelial cell phenotype (Hicks, 1975). If cultured on porous membranes, both cell lines grow into 3 cell layers like in normal murine urothelium *in vivo*. The presence of microvilli in the superficial cells suggests some functional

differentiation in both g/G and NUC-5 cell cultures. The NUC-1 cells are likely to be undifferentiated transformed cells (spindle-cell-like, multilayered and showing disorderly growth).

Ploidy status

Polyploidization is frequently found at an early stage in carcinogenesis (Farber, 1984). Shackney *et al.* (1989) proposed a model in which ploidy changes occur during tumour development: first DNA-diploid tumour cells undergo endoreduplication resulting in tetraploidy and subsequent loss of chromosomes. Cowell and Wigley (1982) and Burholt *et al.* (1989) confirmed this sequence of events during the carcinogenesis of salivary gland and bronchial epithelial cells. In accordance with this model, several studies on TCC have shown that, when TCC cells become DNA-hypotetraploid, they show a higher growth rate *in vitro* and a more aggressive behaviour *in vivo* than DNA-diploid or tetraploid TCC cells (Tribukait *et al.*, 1982; Badalament *et al.*, 1990; Raghavan *et al.*, 1990). Polyploidization of urothelial cells is also a physiological occurrence frequently observed in the superficial umbrella cells of the urothelium (Hicks, 1975). The DNA-tetraploidy of the non-tumorigenic g/G cells supports the idea that g/G cells represent either normal or pre-malignant urothelial cells. The DNA-aneuploid non-tumorigenic NUC-5 cells represent an intermediate position in malignancy. The DNA-aneuploid, tumorigenic and invasive NUC-1 cells show characteristics of highly malignant carcinoma cells.

Growth-factor responsiveness and EGF-R expression

In malignant tumour cells a decreased or altered dependence on growth factors (such as EGF/TGFα) for growth has been observed compared to less malignant tumour cells or normal cells (reviewed by Aaronson, 1991). Both g/G and NUC-5 cells show growth-factor-dependent proliferation. NUC-5 cells differ with respect to their TGFβ-induced proliferation from other normal or pre-malignant epithelial cells. A TGFβ-induced proliferation is not unique for NUC-5 cells since it has also been observed with A431 epidermoid carcinoma cells (Lee *et al.*, 1987). Moreover, TGFβ induces proliferation of NUC-5 cells synergistically with EGF or TGFα, instead of antagonistically as commonly noted for most epithelial cells. These observations suggest an aberration in the TGFβ signalling pathway in NUC-5 cells. This altered response of NUC-5 cells to TGFβ supports the idea of these cells being more transformed than g/G cells. NUC-1 cells show autonomous growth and a poor response to the tested growth factors, which might be associated with a lower growth-factor-receptor expression, or with production of autocrine growth factors.

Messing *et al.* (1987) demonstrated a 6- to 8-fold increase in tritiated thymidine incorporation upon instillation of EGF in rat bladder *in vivo*. Primary cultures of normal mouse urothelium showed a 3- to 6-fold increase in tritiated thymidine incorporation upon exposure to EGF or aFGF *in vitro* (data not shown). These data are in the range of the increase in incorporation (up to 10-fold) we noted in the present study with these growth factors.

With respect to EGF-R expression, previous studies showed an EGF-R expression on cells of the basal and intermediate layers in normal urothelium, on cells of all layers in pre-malignant or malignant human urothelium, and on normal human urothelial cells *in vitro* (Messing *et al.*, 1987; Messing and Reznikoff, 1987). The expression of EGF-R on g/G and NUC-5 cells and concomitant EGF/TGFα response compare with these findings on normal and pre-malignant urothelial cells. Although in most invasive or dedifferentiated TCC cells the EGF-R expression is high, some highly invasive, dedifferentiated TCC have a low EGF-R expression (Messing *et al.*, 1987; Neal *et al.*, 1990). Thus, while NUC-1 cells with few EGF receptors and no response to exogenous EGF-like growth factors fit the model of most autologously active tumour cells, they are only characteristic of a subset of (EGF-R negative) invading human TCC (Neal *et al.*, 1990). At present, we are investigating whether autocrine or intracrine growth factors are involved in the regulation of autonomous proliferation of NUC-1 cells.

The responsiveness of g/G and NUC-5 cells to PDGF-A is consistent with the recent demonstration of PDGF receptors on normal (mammary) epithelial cells and (lung) carcinoma cells (Taverna *et al.*, 1991; Antoniades *et al.*, 1992; Elliott *et al.*, 1992). *In vivo* production of PDGF by stromal cells may provide a paracrine mechanism of regulation of proliferation. Since epithelial cells are also able to produce PDGF-A chain proteins (Bronzert *et al.*, 1990), an autocrine stimulation may also be operating in these cells. A physiological role of PDGF in urothelial cells has yet to be demonstrated.

In conclusion, the morphology of g/G cells, their DNA-tetraploid status, their non-tumorigenicity, and their response to growth factors suggest that they represent urothelial cells at an early stage of carcinogenesis. Their responsiveness to TGFβ, their DNA-aneuploidy and non-tumorigenicity suggest that NUC-5 cells represent an intermediate phase in bladder carcinogenesis. Finally, their tumorigenicity, DNA-aneuploidy and growth-factor-independent growth suggest that NUC-1 cells represent TCC cells of higher malignancy. These 3 cell lines provide a suitable tool for studying various aspects of transitional-cell carcinogenesis. At this moment, we are examining the role of growth factors in physiological processes in transitional epithelium and the role of growth factors in transitional-cell carcinogenesis.

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REFERENCES

AARONSON, S.A., Growth factors and cancer. *Science*, **254**, 1146–1153 (1991).

ANTONIADES, H.N., GALANOPOULOS, T., NEVILLE-GOLDEN, J. and O'HARA, C.J., Malignant epithelial cells in primary human lung carcinomas coexpress *in vivo* platelet-derived growth factor (PDGF) and PDGF receptor mRNAs and their protein products. *Proc. nat. Acad. Sci. (Wash.)*, **89**, 3942–3946 (1992).

BADALAMENT, R.A., O'TOOLE, R.V., KENWORTHY, P., YOUNG, D.C., KEYHANI-ROFAGHA, S., SIMON, J., PEREZ, J.F. and DRAGO, J.R., Prognostic factors in patients with primary transitional cell carcinoma of the upper urinary tract. *J. Urol.*, **144**, 859–863 (1990).

BENRAAD, TH.J. and FOEKENS, J.A., Hydroxylapatite assay to measure epidermal growth factor receptor in human primary breast tumours. *Ann. clin. Biochem.*, **27**, 272–273 (1990).

BRONZERT, D.A., BATES, S.E., SHERIDAN, J.P., LINDSEY, R., VALVERIUS, E.M., STAMPFER, M.R., LIPPMAN, M.E. and DICKSON, R.B., Transforming growth factor-β induces platelet-derived growth factor (PDGF) messenger RNA and PDGF secretion while inhibiting growth in normal human mammary epithelial cells. *Mol. Endocrinol.*, **4**, 981–989 (1990).

BURHOLT, D.R., SHACKNEY, S.E., KETTERER, D.M., POLLICE, A.A., SMITH, C.A., BROWN, K.A., GILES, H.R. and SCHEPART, B.S., Karyotypic evolution of a human undifferentiated large cell carcinoma of the lung in tissue culture. *Cancer Res.*, **49**, 3355–3361 (1989).

CARPENTER, G., Epidermal growth factor: biology and receptor metabolism. *J. Cell Sci. Suppl.*, **3**, 1–9 (1985).

CHODAK, G.W., HOSPELHORN, V., JUDGE, S.M., MAYFORTH, R., KOEPPEN, H. and SASSE, J., Increased levels of fibroblast growth factor-like activity in urine from patients with bladder or kidney cancer. *Cancer Res.*, **48**, 2083–2088 (1988).

CHODAK, G.W., SHING, Y., BORGE, M., JUDGE, S.M. and KLAGSBRUN, M., Presence of heparin binding growth factor in mouse bladder tumors and urine from mice with bladder cancer. *Cancer Res.*, **46**, 5507–5510 (1986).

COWELL, J.K. and WIGLEY, C.B., Chromosome changes associated with the progression of cell lines from preneoplastic to tumorigenic phenotype during transformation of mouse salivary gland epithelium *in vitro*. *J. nat. Cancer Inst.*, **69**, 425–433 (1982).

DE BOER, W.I., REBEL, J.M.J., VERMEY, M. and VAN DER KWAST, T.H., Growth factors and extracellular matrix regulate urothelial cell proliferation *in vitro*. *Urol. Res.*, **20**, 455 (1992).

DUBEAU, L. and JONES, P.A., Growth of normal and neoplastic urothelium and response to epidermal growth factor in a defined serum-free medium. *Cancer Res.*, **47**, 2107–2112 (1987).

FARBER, E., The multistep nature of cancer development. *Cancer Res.*, **44**, 4217–4223 (1984).

FOEKENS, J.A., SIEUWERTS, A.M., STUURMAN-SMEETS, E.M.J., DORSSERS, L.C.J., BERNS, E.M.J.J. and KLIJN, J.G.M., Pleiotropic actions of suramin on the proliferation of human breast cells *in vitro*. *Int. J. Cancer*, **51**, 439–444 (1992).

FRANKS, L.M. and HENZELL, S., The development of "spontaneous" neoplastic transformation *in vitro* of cells from young and old mice. *Europ. J. Cancer*, **6**, 357–364 (1970).

HICKS, R.M., The mammalian urinary bladder: an accommodating organ. *Biol. Rev.*, **50**, 215–246 (1975).

HIRATA, Y. and ORTH, D.N., Concentrations of epidermal growth factor, nerve growth factor, and submandibular gland renin in male and female mouse tissue and fluids. *Endocrinology*, **105**, 1383–1387 (1979).

IOUANNEAU, J., GAVRILOVIC, J., CARUELLE, D., JAYE, M., MOENS, G., CARUELLE, J.-P. and THIERY, J.-P., Secreted or nonsecreted forms of acidic fibroblast growth factor produced by transfected epithelial cells influence cell morphology, motility, and invasive potential. *Proc. nat. Acad. Sci. (Wash.)*, **88**, 2893–2897 (1991).

KIMBALL, E.S., BOHN, W.H., COCKLEY, K.D., WARREN, T.C. and SHERWIN, S.A., Distinct high-performance liquid chromatography pattern of transforming growth factor activity in urine of cancer patients as compared with that of normal individuals. *Cancer Res.*, **44**, 3616–3619 (1984).

KNOWLES, M.A., HICKS, R.M., DAVE, H., HARVEY, A.E., ROBERTS, A.B. and SPORN, M.B., Transforming growth factors induce markers of neoplasia in cultured adult rat bladder. *Carcinogenesis*, **6**, 595–604 (1985).

LEE, K., TANAKA, M., HATANAKA, M. and KUZE, F., Reciprocal effects of epidermal growth factor and transforming growth factor β on the anchorage-dependent and -independent growth of A431 epidermoid carcinoma cells. *Exp. Cell Res.*, **173**, 156–162 (1987).

MESSING, E.M., HANSON, P., ULRICH, P. and ERTURK, E., Epidermal growth factor interactions with normal and malignant urothelium: *in vivo* and *in situ* studies. *J. Urol.*, **138**, 1329–1335 (1987).

MESSING, E.M. and REZNIKOFF, C.A., Normal and malignant human urothelium: *in vitro* effects of epidermal growth factor. *Cancer Res.*, **47**, 2230–2235 (1987).

MUSTOE, T.A., PIERCE, G.F., MORISHIMA, C. and DEUEL, T.F., Growth factor-induced acceleration of tissue repair through direct and inductive activities in a rabbit dermal ulcer model. *J. clin. Invest.*, **87**, 694–703 (1991).

NEAL, D.E., SHARPLES, L., SMITH, K., FENNELLY, J., HALL, R.R. and HARRIS, A.L., The epidermal growth factor receptor and the prognosis of bladder cancer. *Cancer*, **65**, 1619–1625 (1990).

PRIEST, J.H., *Cytogenetics*, pp. 66–67, Lea and Febiger, Philadelphia (1969).

RAGHAVAN, D., SHIPLEY, W.U., GARNICK, M.B., RUSSELL, P.J. and RICHIE, J.P., Biology and management of bladder cancer. *New Engl. J. Med.*, **322**, 1129–1138 (1990).

SHACKNEY, S.F., SMITH, C.A., MILLER, B.W., BURHOLT, D.R., MURTHA, K., GILES, H.R., KETTERER, D.M. and POLLICE, A.A., Model for the genetic evolution of human solid tumors. *Cancer Res.*, **49**, 3344–3354 (1989).

TAVERNA, D., GRONER, B. and HYNES, N.E., Epidermal growth factor receptor, platelet-derived growth factor receptor, and c-erbB-2 receptor activation all promote growth but have distinct effects upon mouse mammary epithelial cell differentiation. *Cell Growth Differ.*, **2**, 145–154 (1991).

TRIBUKAIT, B., GUSTAFSON, H. and ESPOSTI, P.-L., The significance of ploidy and proliferation in the clinical and biological evaluation of bladder tumours: a study of 100 untreated cases. *Brit. J. Urol.*, **54**, 130–135 (1982).

VAN DER KWAST, T.H., VAN ROOY, H. and MULDER, A.H., Establishment and characterization of long-term primary mouse urothelial cultures. *Urol. Res.*, **17**, 289–293 (1989).

YURA, Y., HAYASHI, O., KELLY, M. and OYASU, R., Identification of epidermal growth factor as a component of the rat urinary bladder tumor-enhancing urinary fractions. *Cancer Res.*, **49**, 1548–1553 (1989).

Chapter 6.

HYPERPLASIA OF EPITHELIUM ADJACENT TO TRANSITIONAL CELL CARCINOMA CAN BE INDUCED BY GROWTH FACTORS THROUGH PARACRINE PATHWAYS.

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ABSTRACT

Hyperplasia of transitional cell epithelium adjacent to human transitional cell carcinomas (TCC) is a common finding in pathology. This hyperplasia may be a precancerous aberration. Alternatively, it has been suggested that the hyperplasia is due to paracrine action of tumour-derived growth factors. In this study we tested the latter hypothesis using the mouse tumorigenic TCC cell line NUC-1. Transplantation of NUC-1 tumour cells into the urinary bladder submucosa of syngeneic mice in vivo induced hyperplasia of normal adjacent urothelium in all tested mice. Instead, implantation of normal mouse bladder mucosa did not induce urothelial hyperplasia. In vitro, conditioned medium of NUC-1 cells induced the proliferation of the mouse urothelial cell line g/G, which closely resembles normal urothelial cells. This induction was inhibited by TGFß$_1$. Similarly, TGFß$_1$ inhibited the FGF-1 and FGF-2 induced proliferation of g/G cells. Chemico-physical examination, bioassays with conditioned media, and RNA analysis of NUC-1 cells revealed that these cells predominantly secreted a growth factor with FGF-like properties. These results indicate that epithelial hyperplasia surrounding carcinomas is not necessarily a precancerous aberration, but may result from direct paracrine action of tumour-derived growth factors.

INTRODUCTION

Several morphological aberrations are frequently observed in the human epithelium lining the mucosa adjacent to carcinomas. For example, epithelial hyperplasia represents a rather common finding in a variety of carcinomas, like colon carcinomas [16,19], oesophageal carcinomas [4], and transitional cell carcinoma (TCC) of the urine bladder [2,11]. Its pathogenesis is not clear yet. Two hypotheses have been put forward. According to one hypothesis, the hyperplastic epithelium is a precancerous aberration, which can change into dysplasia and finally to a carcinoma [11]. According to the other hypothesis, hyperplasia does not represent a precancerous stage, but develops as a reaction to the tumour, possibly because of tumour-derived growth factors acting on the surrounding epithelium [19].

Several data implied an important role for growth factors in the process of tumour growth by affecting the proliferation or differentiation of both normal and tumour cells (reviewed by Aaronson [1]). Growth factors can be

synthesized and secreted by both normal cells and tumour cells, and they regulate physiological cellular processes by an autocrine or paracrine pathway. Some growth factors are present in normal urine (e.g. epidermal growth factor (EGF)) [3]. In bladder tumours and in urine of patients with bladder tumours other growth factors, that is transforming growth factor α (TGFα) and fibroblast growth factors (FGFs), have been found [5,6,10,17]. These growth factors are known to affect the proliferation of both transformed and non-transformed urothelial cells in vitro [7,14,22].

Based on experimental work, Sekikawa et al [19] suggested that hyperplasia of colon mucosa adjacent to colon carcinoma could be due to tumour-derived growth factors. In this study we investigated whether hyperplasia of normal urothelial cells adjacent to TCC could indeed be induced by TCC-derived growth factors through paracrine action. Therefore, we first developed an in vivo model to demonstrate the effects in vivo of mouse bladder carcinoma on the surrounding urothelium. To this end, we transplanted a mouse urothelial tumorigenic cell line, designated NUC-1 [7], into the submucosa of the urine bladder of syngeneic mice. Secondly, we determined in an in vitro model the presence of tumour-derived growth factors, that could enhance the growth or proliferation of normal mouse urothelial cells. We studied the NUC-1 cell line for the presence of growth factors, and their effects on the non-tumorigenic mouse urothelial cell line g/G resembling normal mouse urothelial cells [7]. The results from this study show that epithelial hyperplasia surrounding a carcinoma can develop due to tumour-derived growth factors.

MATERIALS AND METHODS

Chemicals

Culture media and supplements were purchased from Flow Laboratories (Zwanenburg, NL) and Sigma (St.Louis, USA), respectively. Human FGF-2 and TGFα were obtained from Oncogene Science (Uniondale, USA), human FGF-1 from Boehringer (Almere, NL), and mouse EGF, endothelial cell-derived growth factor (ECGS), and fibronectin from Sigma (St.Louis, USA). Human transforming growth factor $ß_1$ (TGF$ß_1$) was either obtained from Sigma (St.Louis, USA), or purified from outdated human platelets as described [23]. Tritiated thymidine was purchased from Amersham ('sHertogenbosch, NL).

Cell lines and cultures

Mouse urothelial cell lines NUC-1 and g/G were developed as described

previously [7]. These cells were cultured routinely in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F10 medium, supplemented with 10 % foetal calf serum (FCS), 10 μM HEPES buffer, 100 IU/ml penicillin, 100 μg/ml streptomycin, selenite, transferrin, and 5 μg/ml insulin (standard medium) [7]. Serum-free culture was done in standard medium without FCS, but further supplemented with 0.1 % bovine serum albumin (BSA), putrescin, ethanolamine, spermin, and spermidin as described [7].
A431 carcinoma cells were obtained from Dr. P.M.J.J. Berns (Dept. of Biochemistry, Daniel den Hoed Cancer Centre, Rotterdam, NL). These cells were routinely cultured in RPMI 1640 medium, supplemented with 10 μM HEPES buffer, 10 % FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. ML-CCL64 mink lung carcinoma cells were cultured routinely in DMEM, buffered with 25 mM BES, and supplemented with 5 % FCS, streptomycin and penicillin as outlined above. Foetal bovine heart endothelial (FBHE) cells were routinely cultured in DMEM, supplemented with 10 % FCS.

Transplantation studies

NUC-1 cells were cultured under routine conditions to confluency. After extensive washing with phosphate buffered saline (PBS) pH 7.2, cells were trypsinized, washed with PBS again and injected (1 X 10^6 cells in 100 μl PBS) subcutaneously in normal syngeneic mice. After three weeks, NUC-1 tumours were excised and minced into 1 mm^3 pieces. One piece was transplanted into the submucosa of the bladder of syngeneic mice via a small incision in the muscular layer of the left side of the bladder dome. As a control, 1 mm^3 pieces of normal bladder from syngeneic mice was transplanted similarly.

Collection of conditioned medium

NUC-1 cells were grown in 167 cm^2 culture flasks (Falcon, Lincoln Park, USA) to subconfluency in standard medium. The cells were then washed with PBS, incubated during one hour with serum-free medium, washed again with PBS, and finally incubated during 24 or 48 hours with 35 ml of serum-free medium. After collection, all media were filtered through a 0.2 μm filter, and frozen at - 20° C until use.

Treatment of medium

Heat treatment of medium was done at 65° C for 30 minutes. Acid treatment was done with 1.0 N HCl for one hour, followed by neutralisation

with 0.1 N NaOH. Reduction of medium components was done with 0.1 M dithiothreitol (DTT, Boehringer, Almere, NL) for one hour, followed by dialysis of the medium two times against serum-free medium, and a final filtration through a 0.2 μm filter. Enzymatic digestion of medium proteins was done with 10 μg/ml trypsin for one hour. The trypsin was inactivated by addition of 40 μg/ml trypsin inhibitor. Heparin-binding capacity of the conditioned medium was tested by incubation with heparin-sepharose (Pharmacia, Woerden, NL) during two hours at 4° C. The medium was collected by centrifugation. The heparin-sepharose beads were washed firstly with water and subsequently treated with stepwise increases in the NaCl concentration ranging from 0.15 M to 2.0 M. The eluted fractions were collected by centrifugation at 4° C after each treatment and tested for their ability to induce the proliferation of g/G cells.

Bioassays

Testing effects of growth factors on the proliferation of g/G cells was done as described [7]. In short, cells were first washed with PBS, then incubated during 1 hour in serum-free medium, washed again, and trypsinized. Fibronectin-precoated wells in a 96-multiwell dish (NUNC, Roskilde, DK) were filled with 10,000 viable cells per well in 150 μl serum-free medium. After 16 hours, conditioned medium fractions to be tested were added (50 μl/well). After another 72 hours, cultures were terminated.

The presence of growth factors in NUC-1 conditioned medium was assessed in bioassays with different cell lines. The proliferation of these cell lines is modulated by specific growth factors under defined culture conditions [24]. The proliferation of ML-CCL64 and A431 cells is specifically inhibited by members of the TGFß family and the EGF family, respectively. The proliferation of FBHE cells is specifically enhanced by members of the FGF family.

Bioassays with ML-CCL64 cells, A431 cells, and FBHE cells were done as described previously [24]. In short, A431 cells were grown to subconfluency, rinsed with PBS, trypsinized, counted, and seeded at 2500 viable cells per well in a 96-multiwell dish in 150 μl/well DMEM medium with 5 % FCS. After 16 hours, 50 μl of fractions to be tested were added. Cells were then incubated for another 72 hours. ML-CCL64 and FBHE cells were seeded at 5,000 viable cells per well of a 24-well dish in 1 ml of DMEM with FCS (ML-CCL64) or without FCS (FBHE). After 3 hours, 200 μl of the fractions that were to be tested, was added per well. Cells were then cultured for another 68 hours. In experiments with neutralizing antibodies against $TGF\beta_1$ or $TGF\beta_2$ (British Biotechnology,

Oxford, GB) the fractions were preincubated with 2.0 μg of antibody/ml medium at 37° C before addition to ML-CCL64 cells.

Proliferation

Proliferation was assessed in vitro by [^{3}H]-thymidine labelling using scintillation counting, and in vivo by bromodeoxyuridine (BrdU) labelling for the determination of the labelling index (L.I.) by immunocytochemistry.

For assessing the proliferation in vitro, 0.5 μCi [^{3}H]-thymidine per well was added sixteen hours before termination. Cells in 96-multiwell dishes were rinsed with PBS, trypsinized, and harvested. Tritiated thymidine was counted using a BetaPlate scintillation counter (Wallac-Pharmacia, Woerden, NL). Alternatively, cells in 24-wells dishes were rinsed with PBS, fixed with methanol, lysed in 0.1 M NaOH, and radioactivity in cell lysates was measured in a liquid α,ß scintillation counter. Radioactivity was expressed as counts per minute.

For assessing the proliferation in vivo, mice were injected i.p. with bromodeoxyuridine (BrdU) in PBS (30 mg/kg body weight) one hour before sacrifice. Immunohistochemistry on paraffin-embedded tissue was performed as described previously [18], using the primary anti-BrdU antibody IIB5 (kindly donated by Prof.Dr. F.C.S. Ramaekers, University of Limburg, Maastricht, NL). The L.I. was calculated as the relative number of BrdU positive cells per 100 adjacent cells in the basal urothelial cell layer (normal) or 200 adjacent cells in the first two basal cell layers (hyperplastic). The L.I. is given as the mean L.I. ± standard deviation.

RESULTS

In vivo model.

After 1, 2, and 3 weeks, five mice were sacrificed and urine bladders were examined. No tumours were found in mice in which normal urine bladder was transplanted. The transplanted normal bladder had largely degenerated within one week. Hyperplasia of the urothelium was not noted (Figure 1A) and BrdU incorporation was low in the overlying urothelium (Table 1). In contrast, at all three time intervals after transplantation of NUC-1 tumour pieces, the urothelium overlying the transplants was hyperplastic, i.e. 5 or more cell layers (Figure 1B). The hyperplastic urothelium also showed high BrdU incorporation, particularly in basal cell layers (Table 1). No nuclear atypia was noted in the hyperplastic urothelium. The urothelium lining the bladder lumen opposite to the

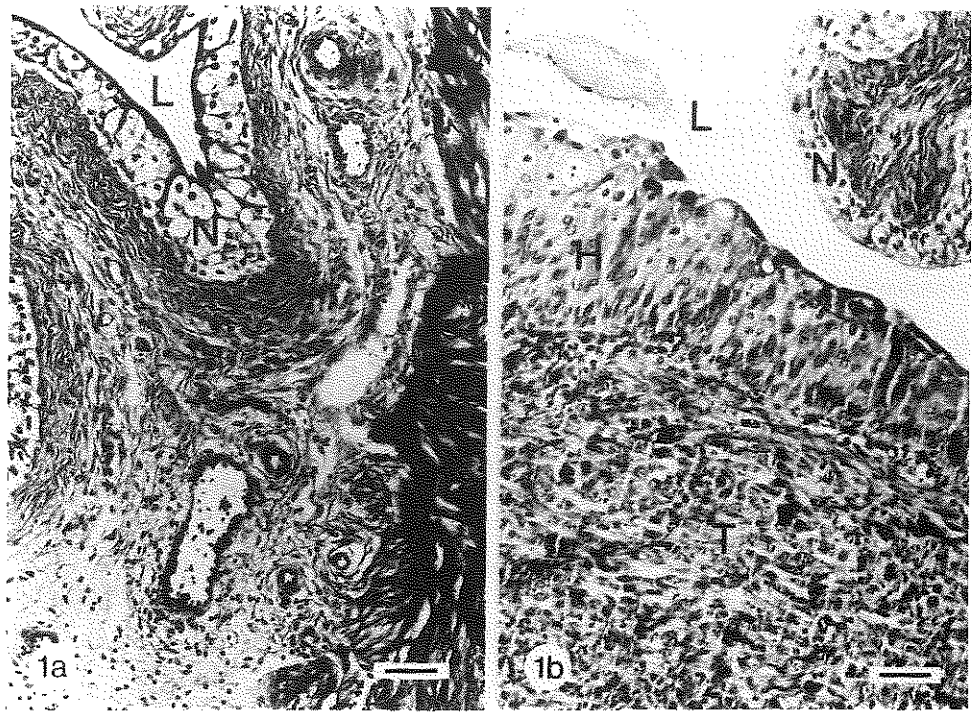

Figure 1. Photomicrograph of haematoxylin-stained paraffin-embedded sections of (A) normal mouse urothelium and (B) a tumour of NUC-1 mouse urothelial cells one week after trans-plantation into the bladder submucosa of a syngeneic mouse. A. Normal, non-hyperplastic urothelium adjacent to the region of degenerated, transplanted normal bladder. B. Hyperplastic urothelium overlying the NUC-1 tumour, and non-hyperplastic urothelium at the opposed bladder wall. H = hyperplastic urothelium, N = normal urothelium, L = lumen of bladder, T = NUC-1 tumour. Magnification = 200 X; bar = 50 μm.

hyperplastic urothelium exhibited a normal appearance in 5 out of 5 mice after one week (Table 1), and showed a normal L.I..

Chemico-physical analysis and bioassays.

The next step was to find out if and which NUC-1 derived growth factors might be involved in the development of hyperplasia. First, we examined whether NUC-1 cells produced and secreted growth stimulatory factors. Therefore, a chemico-physical analysis of serum-free conditioned medium of NUC-1 cells was performed. The conditioned medium was tested in bioassays

Table 1. Occurence of hyperplasia in the in vivo transplantation model.

weeks	tumours	hyperplasia	number of cell layers	L.I. (%) hyperplastic	L.I. (%) normal
transplanted NUC-1 tumours					
1	5	5*	6 - 10	9.6 ± 5.6	0.7 ± 0.7
2	5	3**	5	20.7 ± 8.5	1.6 ± 0.1
3	5	4	5 - 10	11.5 ± 4.7	4.4 ± 0.5
transplanted normal bladder					
1	0	0	3		0.6 ± 0.6
2	0	0	3		0.2 ± 0.2
3	0	0	3		0.5 ± 0.5

Table 1. In vivo data on transplanted tissue into bladder submucosa. The number of tumours out of 5 animals is given at the indicated time interval after transplantation (weeks). The number of mice in which hyperplasia adjacent to the tumour was observed, is also given, as well as the number of cell layers in the hyperplastic urothelium. The proliferation was expressed as the mean labelling index (L.I.) ± standard deviation (n = 5). In 2 out of 5 mice (*) and in 1 out of 5 mice (**) the overlying urothelium was completely replaced by tumour cells.

with g/G cells. As presented in figure 2A, conditioned medium of NUC-1 induced the proliferation of g/G cells, which could be inhibited by exogenous addition of TGFß$_1$. Treatment with trypsin or acid completely abolished the activity of the NUC-1 derived conditioned medium, and treatment with heat partially reduced the enhanced proliferation. Treatment with DTT only slightly affected the enhanced proliferation of g/G cells. This slight decrease in proliferation might be due to the dialysis step following DTT treatment. The results indicate that at least a DTT-stable polypeptide growth factor is present in the NUC-1 conditioned medium. The sensitivity to heat and acid treatment might indicate that the proliferation inducing activity is a heat and acid labile protein. On the other hand, the possibility that latent NUC-1 derived TGFß is activated upon heat or acid treatment, resulting in an inhibition of proliferation, cannot be excluded.

Next, we investigated the effect of different growth factors on proliferation of g/G cells under serum-free conditions. Figure 2B shows that EGF, TGFα, FGF-1, and FGF-2 could induce the proliferation of g/G cells. This growth factor induced proliferation could be inhibited by addition of TGFß$_1$.

A further characterization of the proliferation-inducing activity was made

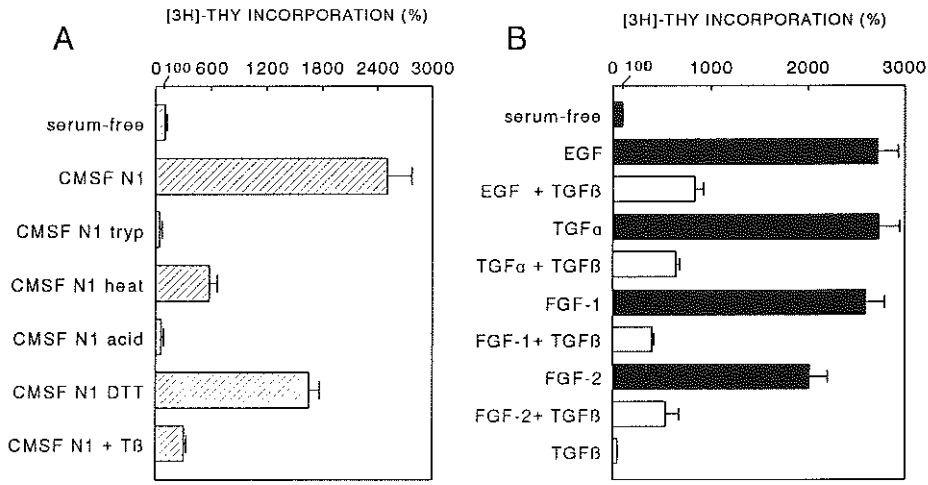

Figure 2. [^{3}H]-thymidine incorporation of g/G cell to (A) serum-free conditioned medium of NUC-1 cells and (B) growth factors in serum-free medium. A. Proliferation in the presence of serum-free conditioned medium of NUC-1 with or without 1 ng/ml TGFß$_1$, or pretreated under the indicated conditions. Incorporation in serum-free medium alone was taken as 100 %. Tryp = trypsin-treated, heat = heat-treated, acid = acid-treated, DTT = DTT-treated, Tß = TGFß$_1$. B. Proliferation in the presence of the indicated growth factors alone (closed bars) or in the presence of 1 ng/ml TGFß$_1$ (open bars). Incorporation in serum-free medium alone was taken as 100 %. Growth factor concentrations: 1 ng/ml (EGF, TGF*α*, TGFß$_1$) or 10 ng/ml (FGF-1, FGF-2).

using a series of growth factor specific bioassays. Bioassays with ML-CCL64 cells revealed TGFß-like activity in NUC-1 derived conditioned medium that was heat-pretreated for activation of TGFß, but this activity could not be neutralized by specific antibodies against TGFß$_1$ or TGFß$_2$, nor by combinations of these antibodies (Figure 3A). Untreated NUC-1 conditioned medium was not active in the ML-CCL64 bioassay (data not shown). Bioassays with A431 indicated that the serum-free conditioned medium of NUC-1 cells did not contain detectable EGF-like growth factors (Figure 3A). In particular, the resistance to DTT treatment excludes the involvement of growth factors of which the biological activity is dependent on intact disulphide bonds. In order to test the possibility that the NUC-1 derived growth factor polypeptide was an FGF-like protein, which is DTT stable, FGF-specific bioassays with FBHE cells were performed and the heparin-binding capacity was determined. Figure 3A shows that untreated conditioned medium of NUC-1 stimulated the proliferation of FBHE cells by 5 times. If the conditioned medium of NUC-1 was treated with heparin-

sepharose, then washed with water, and subsequently eluted with increasing salt concentrations, the stimulatory protein was mainly contained in the 0.15 M NaCl fraction (Figure 3B). The first fraction which was eluted in the absence of salt, did not induce the proliferation (Figure 3B). This indicates the presence of a protein in conditioned medium of NUC-1 that binds specifically to heparin though with low affinity. Analysis of Western blots did not reveal any FGF-2-like molecule in NUC-1 conditioned medium. In line with this result, Northern blot analysis of poly A^+ RNA from NUC-1 cells did not demonstrate detectable FGF2 expression. In addition, we failed to detect expression of FGF-1, FGF-3, and FGF-4 RNA in NUC-1 cells (data not shown).

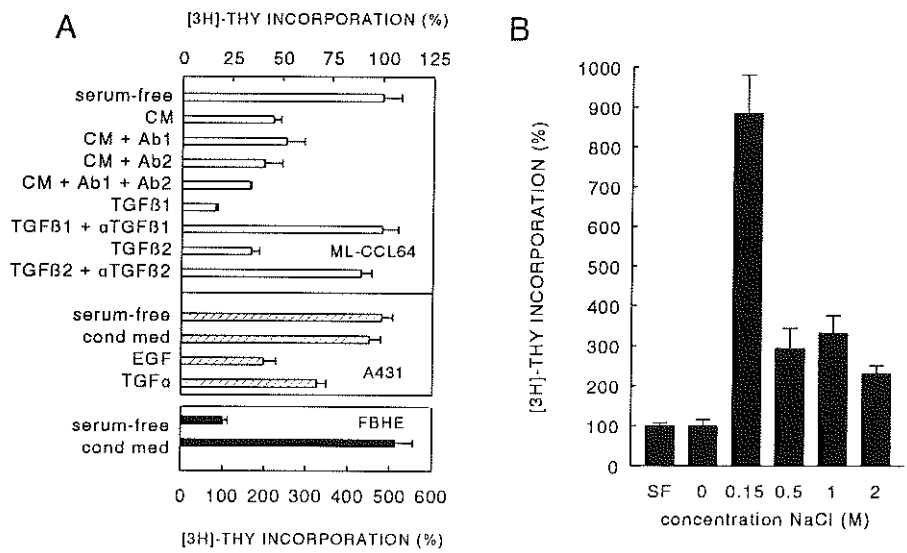

Figure 3. Bioassays with ML-CCL64, A431, FBHE, or g/G cells. A. Incorporation of tritiated thymidine into the indicated cell lines in the presence of serum-free conditioned medium of NUC-1 (CM), or in the presence of 0.88 ng/ml $TGFß_1$ or $TGFß_2$ pretreated or not with antibody against $TGFß_1$ (Ab1) or $TGFß_2$ (Ab2) (ML-CCL64 assay), 1 ng/ml EGF or TGFα (A431 assay), or 15 μg/ml ECGS (FBHE assay). Conditioned medium of NUC-1 cells was heat-pretreated before use in the bioassay with ML-CCL64 cells; for use in the other bioassays, the NUC-1 conditioned medium was not pretreated. Proliferation of ML-CCL64 and A431 cells are on the upper axis, and of FBHE cells on the lower axis. Incorporation in the presence of non-conditioned serum-free medium alone was taken as 100 %. ECGS (15 μg/ml) induced the proliferation of FBHE cells approximately 20 times. B. Incorporation of [^{3}H]-thymidine into g/G cells in the presence of heparin-sepharose treated serum-free conditioned medium of NUC-1 cells fractionated with increasing salt concentrations. Final dilution of conditioned medium fractions as tested on g/G cells was 1:8. Incorporation in the presence of serum-free medium alone (SF) was taken as 100 %.

DISCUSSION

Several studies on human transitional cell carcinomas (TCC) revealed hyperplasia of the normal urothelium adjacent to the TCC [2,11]. This hyperplasia of adjacent urothelium has also been observed in experimentally induced urothelial carcinomas in the rat [8]. It was hypothesized that this hyperplasia represented a precancerous stage of urothelium [11]. Similarly, several authors considered hyperplasia adjacent to a colon carcinoma as a precancerous alteration [9,16]. This hypothesis was disputed by Sekikawa et al [19]. Using transplantations of colon carcinoma cells in mouse colon in vivo, they demonstrated alterations in mucin expression, morphology, and BrdU incorporation only in the mucosa overlying the tumour, but not in the remote mucosa or in their control animals transplanted with fibroblasts. They hypothesized the involvement of tumour-derived growth factors in the development of this hyperplasia.

The data of the present study on transitional cell hyperplasia of the bladder demonstrated that only epithelium overlying the tumour was hyperplastic and showed a high proliferation. Urothelium opposite or remote from the tumour, as well as urothelium of control transplanted mice did not show hyperplasia or a high proliferation. Bioassays with A431 cells, the lack of detectable TGFα RNA and EGF protein expression in NUC-1 cells (data not shown), and the DTT stability excluded the identification of the NUC-1-derived, growth stimulatory protein as an EGF-like growth factor. Although bioassays with ML-CCL64 cells indicated that NUC-1 cells secreted a TGFß-like factor, this activity could not be neutralized by specific antibodies against TGFß$_1$ or TGFß$_2$. However, other members of the TGFß family, like TGFß$_3$, could also be produced by NUC-1 cells. The data from Figure 2A indicate that the NUC-1 stimulating activity disappears after heat or acid treatment of conditioned medium. This might be due to inactivation of the stimulatory protein, but also to activation of TGFß$_3$. Because neutralizing antibodies against TGFß$_3$ were not available, we were unable to check for the presence of TGFß$_3$ in the NUC-1 derived conditioned medium. The data of the bioassays with g/G cells indicated that the growth stimulating effect of the NUC-1-derived factor can be inhibited by TGFß, as is also the case with the growth stimulating factors EGF and FGF (Figure 2). The heparin-binding data indicate that the major fraction of growth stimulating factors binds heparin with low affinity. RNA analysis of NUC-1 cells failed to detect FGF-1, FGF-2, FGF-3, and FGF-4. Of other FGFs, FGF-5 binds

heparin with high affinity [25], and FGF-7 is a non-epithelial cell derived FGF [8]. These data may exclude the identification of our growth stimulating factor as one of these FGFs. Little is known yet about FGF-6, FGF-8, and FGF-9 with respect to epithelial cells.

Several data support the role of TCC-derived growth factors in hyperplastic development. Both TGFα and FGF-like (FGF-1 or FGF-2) growth factors have been detected in the urine of patients with TCC [5,6,10]. Other studies demonstrated an enhanced expression of FGF-1 proteins [17], FGF-5 RNA expression [25], and co-amplification of *FGF-3* and *FGF-4* genes [20] in human TCCs. Normal urine and urothelium contains EGF in high concentrations and only small amounts of FGF-1 or FGF-2 [3,5,6,17]. The present study and previous studies showed that these EGF-like and FGF-like growth factors could induce proliferation of non-tumorigenic transitional epithelial cells in vitro and in vivo [7,13]. Supporting evidence for the involvement of FGF-like factors in hyperplasia of epithelium was given by Muller et al [15], who showed that expression of the *FGF-3* gene in prostate or mammary gland epithelium of *FGF-3* transgenic adult mice resulted in epithelial hyperplasia in these organs. Hence, the observed hyperplasia of urothelium adjacent to TCC in vivo may be due to a direct, paracrine effect of TCC-derived FGF on the normal urothelial cells. However, we do not know yet whether the NUC-1-derived factor is indeed FGF-like.

Hyperplasia of epithelium adjacent to primary carcinomas has also been found in the oesophagus [4]. A striking finding is that approximately 50 % of all primary oesophageal carcinomas have a coamplification of the *FGF-3* and *FGF-4* genes [21]. This indicates a more general involvement of FGF-like growth factors in the development of epithelial hyperplasia adjacent to carcinomas.

In conclusion, in this study we obtained evidence for the induction of hyperplasia of urothelium surrounding tumorigenic urothelial NUC-1 cells transplanted into the bladder wall. We also demonstrated that mouse tumorigenic urothelial NUC-1 cells produced and secreted a protein in vitro which was DTT stable, bound to heparin, and stimulated the proliferation of FBHE cells, which respond specifically to FGFs. The present data support the hypothesis that hyperplasia in bladder biopsis of patients with TCC does not necessarily reflect a precancerous lesion prone to the development of a new TCC. The observed hyperplasia may be due to tumour-derived growth factors which have some chemico-physical and biological properties of FGFs. A full characterization of the TCC-derived growth stimulating factor(s) awaits further

study.

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REFERENCES

1. Aaronson AS. Growth factors and cancer. *Science* 1991; **254**: 1146-1153.

2. Brünger R, Schubert GE. Urothelial histopathology surrounding bladder carcinoma. *Eur. Urol.* 1987; **13**: 156-162.

3. Carpenter G. Epidermal growth factor: biology and receptor metabolism. *J. Cell. Sci. Suppl.* 1985; **3**: 1-9.

4. Chang F, Syrjänen S, Shen Q, Ji H, Syrjänen K. Human papillomavirus (HPV) DNA in oesophageal precancer lesions and squamous cell carcinomas from China. *Int. J. Cancer* 1990; **45**:21-25.

5. Chodak GW, Hospelhorn V, Judge SM, Mayforth R, Koeppen H, Sasse J. Increased levels of fibroblast growth factor-like activity in urine from patients with bladder or kidney cancer. *Cancer Res.* 1988; **48**: 2083-2088.

6. Chopin DK, Caruelle J-P, Colombel M, Palcy S, Ravery V, Caruelle D, Abbou CC, Barritault D. Increased immunodetection of acidic fibroblast growth factor in bladder cancer, 8detectable in urine. *J. Urol.* 1993; **150**: 1126-1130.

7. De Boer WI, Rebel JMJ, Foekens JA, Vermey M, Van der Kwast TH. Characterization of mouse urothelial cell lines in different phases of transitional cell carcinogenesis. *Int. J. Cancer* 1993; **54**: 1022-1027.

8. Finch PW, Rubin JS, Miki T, Ron D, Aaronson SA. Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science* 1989; **245**:752-755.

9. Greaves P, Filipe MI, Branfoot AC. Transitional mucosa and survival in human colorectal cancer. *Cancer* 1980; **46**: 764-770.

10. Kimball ES, Bohn WH, Cockley KD, Warren TC, Sherwin SA. Distinct high-performance liquid chromatography pattern of transforming growth factor activity in urine of cancer patients as compared with that of normal individuals. *Cancer Res.* 1984; **44**: 3613-3619.

11. Koss LG. Tumors of the urinary bladder. Washington: Armed Forces Institute of Pathology, 1975, Vol 11.

12. Koss LG, Lavin P. Studies of experimental bladder carcinoma in Fischer 344

female rats. I. Induction of tumors with diet low in vitamin B6 containing N-2-fluorenylacetamide after single dose of cyclophophamide. *Natl. Cancer Inst. Monogr.* 1971; **46**: 585-595.

13. Messing EM, Hanson P, Ulrich P, Erturk E. Epidermal growth factor- interactions with normal and malignant urothelium: in vivo and in situ studies. *J. Urol.* 1987; **138**: 1329-1335.

14. Momose H, Kaknuma H, Shariff SY, Mitchell GB, Rademaker A, Oyasu R. Tumor-promoting effect of urinary epidermal growth factor in rat urinary bladder carcinogenesis. *Cancer Res.* 1991; **51**: 5487-5490.

15. Muller WJ, Lee FS, Dickson C, Peters G, Pattengale P, Leder P. The *int-2* gene product acts as an epithelial growth factor in transgenic mice. *EMBO J.* 1990; **9**: 907-913.

16. Penelope AD, Filipe MI. An ultrastructural and histochemical study of the mucous membrane adjacent to and remote from carcinoma. *Cancer* 1976; **37**: 2388-2398.

17. Ravery V, Jouanneau J, Gil-Diez S, Abbou CC, Caruelle J-P, Barritault, Chopin DK. Immunohistochemical detection of acidic fibroblast growth factor in bladder transitional cell carcinoma. *Urol. Res.* 1992; **20**: 211-214.

18. Schutte B, Reynders MMJ, Van Assche CLMVJ, Hupperets PSGJ, Bosman FT, Blijham GH. Studies with anti-bromodeoxyuridine antibodies. *Cytometry* 1987; **8**: 372-376.

19. Sekikawa K, Arends JW, Verstijnen K, Ten Kate J, Schutte B, Bosman FT. Colonic mucosal chamges in nude mice associated with orthotopic xenografts of human colon cancer cells. *Virchow's Archiv A* 1990; **416**: 393-396.

20. Theillet C, Le Roy X, De Lapeyrière O, Grosgeorges J, Adnane J, Raynaud SD, Simony-Lafontaine J, Goldfarb M, Escot C, Birnbaum D, Gaudray P. Amplification of *fgf*-related genes in human tumors: possible involvement of *HST* in breast carcinomas. *Oncogene* 1989; **4**: 915-922.

21. Tsuda T, Tahara E, Kajiyama G, Skamoto H, Tereda M, Sugimura T. High incidence of coamplification of *hst-1* and *int-2* genes in human esophageal carcinomas. *Cancer Res.* 1989; **49**: 5505-5508.

22. Vallés AM, Boyer B, Badet J, Tucker GC, Barritault D, Thierry JP. Acidic fibroblast growth factor is a modulator of epithelial plasticity in a rat bladder carcinoma cell line. *Proc. Natl. Acad. Sci. (USA)* 1990; **87**: 1124-1128.

23. Van den Eijnden-van Raaij AJM, Koorneef I, Van Zoelen EJJ. A new method for high yield purification of type beta transforming growth factor from human platelets. *Biochem. Biophys. Res. Comm.* 1988; **157**: 16-23.

24. Van Zoelen EJJ. The use of biological assays for detection of polypeptide growth factors. *Progress Growth Factor Res.* 1990; **2**: 131-152.

25. Zhan X, Bates B, Hu X, Goldfarb M. The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. *Mol. Cell. Biol.* 1988; **8**: 3487-3495.

Chapter 7. SUMMARY AND CONCLUDING REMARKS

The urothelium lining the lower urinary tract is often exposed to toxic agents or to objects causing urothelial damage. In other epithelia growth factors play a major role in the maintenance and repair through paracrine and autocrine actions. As yet, little is known about the role of growth factors in bladder epithelium. This issue is addressed in this thesis. The direct effects of in vitro exposure to growth factors were studied on mouse urothelial cell lines and on primary, organoid-like cultures of mouse urothelium. The localization and possible function of growth factors was further substantiated by in vivo experiments with mice either under physiological (i.e. wound healing) or under neoplastic (i.e. transitional cell carcinoma growth) conditions.

§ 7.1 Primary urothelial cultures as a model for physiological urothelial growth.

The first part of this thesis deals with possible functions of a selected set of growth factors in the physiology of mouse urothelium. In order to study effects of growth factors on urothelial cells without the complicating interference of other cell types, organotypic cultures of mouse urothelium were needed. The urothelial cell lines as described in chapter 5 were not suitable as they did not develop a normal stratification. However, as described in chapter 2, primary cultures of mouse urothelium grown on Cyclopore membranes morphologically mimick mouse urothelium in vivo. In this system, relevant parameters for proliferation, differentiation, and culture expansion can be evaluated on a single culture.

In chapter 3, the effects of different growth factors on these parameters are described for primary cultures reflecting intact or regenerating urothelium in vivo. Both FGF-1 and EGF could enhance proliferation and migration of urothelial cells, but not differentiation. In contrast, TGFß$_1$ induced terminal differentiation and apoptosis of urothelial cells and inhibited proliferation. PDGF-BB enhanced the migration of urothelial cells slightly, while FGF-2 and PDGF-AA did not show any effect. This suggests that in murine urothelium FGF-1, EGF, and TGFß$_1$ are more relevant growth factors than FGF-2 or both PDGFs.

In chapter 4 the in vitro observations made in chapter 3 and 5 were compared with those in an in vivo mouse model for regeneration of acutely injured urothelium. The spatio-temporal RNA and/or protein expression of the

selected growth factors and some of their receptors was studied by in situ hybridization or immunocytochemistry. Changes in the RNA and protein levels were only observed in the urothelium, except for TGFß and its receptor. Migrating and proliferating urothelial cells displayed an increased FGF-1 RNA expression. Similarly, $TGF\beta_1$ RNA expression was enhanced especially during the migration and differentiation phase of the urothelium. Concomittant with the TGFß RNA (and protein) expression, the expression of TGFß type II receptor proteins was also enhanced in the regenerating urothelium. FGF-2 expression was low to undectable in urothelial cells. These data correlate well with our observations regarding the effects of the growth factors on primary urothelial cultures (chapter 3). IGF type I receptor and IGF-II RNA were predominantly expressed during the urothelial migration and proliferation phase. IGF-II may affect urothelial proliferation by enhancing the progression through the cell cycle as indicated in chapter 5. IGFBP-2 was expressed constitutively in the urothelium. Although a high constitutive PDGF-A RNA expression was observed, no PDGF receptor RNA expression was detectable. Taken together, these data indicate that the acute damaged mouse urothelium supports its own regeneration through at least FGF-1, $TGF\beta_1$, and IGF-II. However, our observations do not entirely exclude paracrine involvement of mesenchyme-derived growth factors on urothelial regeneration.

The combination of the in vitro and in vivo data indicates that FGF-1, $TGF\beta_1$, IGF-II are relevant for the physiology of the murine urothelium in vivo.

§ 7.2 Urothelial cell lines as a model for normal or aberrational urothelial growth.

In chapter 5, the establishment and characterization of three mouse urothelial cell lines were described. These cell lines are considered each to represent different phases in transitional cell carcinogenesis. One of these cell lines, designated g/G, closely resembles normal mouse urothelial cells with respect to e.g. morphology and response to growth factors. The second cell line, designated NUC-5, differs from g/G in being more transformed and showing a TGFß-stimulated proliferation. The third cell line, designated NUC-1, is tumorigenic and represents a subgroup of malignant, invasive TCC cells with respect to morphology, chromosome number, and absence of response to growth factors. Hence, the progression of urothelial cells towards neoplasia tends to be accompanied by an aberrant response to growth factors. In support of this observation, other authors found that transformed epithelial cells in vitro

responded different to growth factors, including TGFß, compared with their normal counterparts (Lee et al, 1987; Terzaghi-Howe, 1989; Valverius et al, 1989). This may be due to e.g. an altered signal transduction pathway (Valverius et al, 1989).

In chapter 6, we examined whether hyperplasia of mouse urothelium adjacent to TCC could be due to tumour-derived growth factors. The tumorigenic NUC-1 cell line was used in an in vivo model for mouse TCC. Urothelial cells overlying the NUC-1 implanted tumour cells indeed showed hyperplasia in vivo. Several in vitro assays were used to examine whether NUC-1 cells produce growth factors capable of stimulating the proliferation of urothelial cells. Both chemico-physical analysis and the bioassays with serum-free conditioned medium of NUC-1 cells indicate that one or more NUC-1 derived growth factors with FGF-like properties are responsible for stimulating the proliferation of the g/G cell line. These data support the idea that hyperplasia of urothelium adjacent to TCC can be attributed to the paracrine action of tumour-derived growth factors rather than being a preneoplastic lesion.

As described in chapter 5 and 6, growth factors which play a role during urothelial wound healing can also affect the proliferation of TCC cells. Though, the response to these growth factors can differ from normal cells due to e.g. an altered expression of growth factors or their receptors (c.f. the low EGF receptor expression in and the non-responsiveness to EGF/TGFα of the tumorigenic NUC-1 cells, or the TGFß-induced proliferation of NUC-5 cells, with that of g/G cells). Such an alteration may result in a selective growth advantage for TCC cells.

§ *7.3 Implications and relevance of the data.*

The present in vivo model for mouse urothelial regeneration differs from wound healing models with murine or porcine skin epithelium with respect to the depth of the wound. It should be realized that in our urothelial wounding model only superficial lesions were induced and not a severe damage to the submucosa. In contrast, a great extent of mesenchymal repair was observed in skin wound healing models. This was parallelled by a larger modulation in expression of growth factors and/or their receptors in the regenerating dermis, as demonstrated for TGFß$_1$, PDGF-B, and PDGF type ß receptor (Antoniades et al, 1991; Levine et al, 1993).

Other growth factors, like FGF-7, may also be involved in wound healing.

Werner et al (1992) observed that the FGF-7 mRNA expression is prominently enhanced within the injured skin dermis. Moreover, the FGF-7 receptor mRNA was detected specifically in epidermal cells (Werner et al, 1992). This suggests that also FGF-7 has a function during epithelial wound healing. FGF-7 mRNA was also found in normal human bladder submucosa while its receptor mRNA can be detected in intact human urothelium (Radvanyi et al, 1993). These observations are in line with a function for FGF-7 in the physiology of bladder urothelium. Furthermore, EGF is present in the urine and stimulates proliferation and migration of murine urothelial cells (Chapter 2 and 5). This suggests a role for EGF in the urothelium in vivo. Though, Hirao et al (1980) demonstrated that heterotopic rat bladder in vivo regenerates in the absence of EGF in the bladder lumen. Hence, future studies should point out whether other known growth factors, like EGF and FGF-7, are also important in the biology of bladder urothelium.

Table 1. Implication of growth factors/receptors in the progression of human TCC.

	expression	prognostic impact	source of specimen
c-erbB-1	+ (p)	+	TCC
c-erbB-2	+ (p)	+	TCC
TGFß$_1$	— to + (R)	?	TCC
TGFß type V rec	— (L)	?	epithelial cell lines **
type I IGF rec	+ (L)	+	urothelial cell lines **
FGF-1	+ (p)	+	TCC
FGF-7 rec	0 to — (R)	+	TCC *

Table 1. Literature outline of the generally observed trend in aberrant expressions of growth factors or growth factor receptors in human TCCs, as compared with normal human urothelium. Expressions were determined at either protein (p) or mRNA (R) levels, or via ligand binding (L). The expression levels in the tumour cells were either decreased (—), increased (+), or remained unchanged (0). Prognostic impact: this column indicates whether expression of the growth factor or growth factor receptor is associated with tumour progression (+). The TGFß type V receptor expression has not been investigated in human TCC. * = data from one study; ** = data from one ligand binding study.

A further characterization of mouse TCC-derived growth factors, as indicated in chapter 6, may also lead to the identification of other growth factors relevant in the biology of urothelium. Additional evidence for the role of all these growth factors in the biology of mouse urothelium may be obtained by in vivo mouse models with specific local delivery of growth factors.

The in vivo relevance for a physiological function in human urothelium remains to be established for each of the growth factors examined in this thesis. For example, according to some authors normal human urothelial cells in vitro do not respond to EGF although they express EGF receptors (Reznikoff and Messing, 1987; Messing and Reznikoff, 1987). The expression patterns of some growth factors and/or their receptors in human urothelium and human TCC have been investigated previously. Frequently, human TCC show aberrations in these expression patterns (Table 1).

§ *7.4 Future directions.*

The in chapter 2 described in vitro model for mouse urothelium in vivo may be relevant for studies on the plasticity of the urothelium: e.g. how is the fast regeneration of damaged urothelium regulated, how can the regeneration process be disturbed, and which factors may be curative for a disturbed regeneration.

Also referring to the latter question, the model may be useful in the research on pharmaca, including growth factors and their antagonists, and extracellular matrix proteins. For example, several in vivo studies showed that application of one or more growth factors to injured skin epithelium increases the regeneration process (Lynch et al, 1989; Mustoe et al, 1991). Based on our results obtained with murine urothelium, we hypothesize that intravesical application of FGF-1, TGFß$_1$, and/or IGF-II will enhance the urothelial regeneration in vivo. In vivo treatment with these factors may prove to be an advantage e.g. in slowly regenerating urothelium.

Furthermore, it will be important to examine the difference in response to growth factors in both normal human urothelium and human TCC of different stage and grade. These studies may provide a basis for explaining differences in the behaviour of human TCCs compared with normal human urothelium, and may also provide a basis for therapeutic experiments to treat human TCCs with growth factors and/or their antagonists .

The final conclusions of this thesis are:

1) our organotypic in vitro model for mouse urothelium is relevant for mouse urothelium in vivo;

2) in mouse urothelium in vitro TGFß$_1$ induces terminal differentiation; FGF-1 and EGF stimulate the proliferation and migration; PDGF-BB slightly enhances the expansion of the culture; and FGF-2 and PDGF-AA have no effects. The RNA/protein expression data of urothelial wound healing in vivo in mice correlate with the in vitro data, suggesting that these growth factors have similar specific functions in vivo. We did not notice major differences in growth factor functions and expression patterns as compared with other epithelia;

3) increasingly transformed urothelial cells display a qualitatively different response to TGFß, i.e. growth stimulation, and are less dependent on growth factors for their growth;

4) tumour-derived growth factor(s) with FGF-like properties may induce hyperplasia of the urothelium adjacent to TCC.

§ *7.5 REFERENCES.*

1 Antoniades HN, Galanopoulos T, Neville-Golden J, O'Hara CJ: Malignant epithelial cells in primary human lung carcinomas coexpress *in vivo* platelet-derived growth factor (PDGF) and PDGF receptor mRNAs and their protein products. Proc. Natl. Acad. Sci. USA 89, 1992:3942-3946.

2 Hirao Y, Izuma K, Oyasu R: The effects of normal rat urine on mucosal regeneration in heterotopic urinary bladder. Lab. Invest. 42, 1980:76-84.

3 Lee K, Tanaka M, Hatanaka M, Kuze F: Reciprocal effects of epidermal growth factor and trasnforming growth factor ß on the anchorage-dependent and -independent growth of A431 epidermoid carcinoma cells. Exp. Cell Res. 173, 1987:156-162.

4 Levine JH, Moses HL, Gold LI, Nanney LB: Spatial and temporal patterns of immunoreactive transforming growth factor beta 1, beta 2, and beta 3 during excisional wound repair. Am. J. Pathol. 143, 1993:368-380.

5 Lynch SE, Colvin RB, Antoniades HN: Growth factors in wound healing. Single and synergistic effects on partial thickness porcine skin wounds. J. Clin. Invest. 84, 1989:640-646.

6 Messing EM, Reznikoff CA: Normal and malignant human urothelium: *in vitro* effects of epidermal growth factor. Cancer Res. 47, 1987:2230-2235.

7 Mustoe TA, Pierce GF, Morishima C, Deuel TF: Growth factor-induced acceleration of tissue repair through direct and inductive activities in a rabbit dermal ulcer model. J. Clin. Invest. 87, 1991:694-703.

8 Radvanyi F, Gil Diez S, Delouvée A, Thiery JP, Abbou C, Chopin D: Loss of KGF receptor expression in the aggressive transitional cell carcinoma (TCC) of the bladder. Urol. Res. 21, 1993:443.

9 Reznikoff C, Loretz L, Pesciotta D, Oberley T, Ignjatovic M: Growth kinetics and differentiation in vitro of normal human uroepithelial cells on collagen gel substrates in defined medium. J. Cell. Physiol. 131, 1987:285-301.

10 Terzaghi-Howe M: Changes in response to, and production of, transforming

growth factor type ß during neoplastic progression in cultured rat tracheal epithelial cells. Carcinogenesis 10, 1989:973-980.
11 Valverius EM, Walker-Jones D, Bates SE, Stampfer MR, Clark R, McCormick F, Dickson RB, Lippman ME: Production of and responsiveness to transforming growth factor-ß in normal and oncogene-transformed human mammary epithelial cells. Cancer res. 49, 1989:6269-6274.
12 Werner S, Peters KG, Longaker MT, Fuller-Pace F, Banda MJ, Williams LT: Large induction of keratinocyte growth factor expression in the dermis during wound healing. Proc. Natl. Acad. Sci. USA 89, 1992:6896-6900.

SAMENVATTING

De urineblaas is een dynamisch orgaan dat als reservoir voor de urine dient. Morfologisch bestaat het uit een spierwand aan de buitenzijde, een stromale laag, en de epitheliale cellaag dat aan het lumen grenst. Het overgangscel epitheel (ook urotheel genoemd) bestaat uit 3 (muis) of meer (mens) cellagen. De cellaag die aan het lumen grenst, wordt de paraplulaag genoemd, en is het meest gedifferentieerd. De urotheel cellaag is niet doorlaatbaar voor urine en vormt een barrière voor het lichaam. In tegenstelling tot andere epithelia zoals van darm en huid, worden de urotheelcellen na lange tijd vervangen door nieuwe urotheel cellen. Als de urotheellaag verwond wordt, dan neemt de delingsaktiviteit en de migratie enorm toe wat resulteert in een snel herstel van het urotheel en zijn functie. Diverse in vitro studies met epitheel- en andere cellen hebben laten zien dat groeifactoren de celdeling (of proliferatie), migratie, differentiatie en/of celdood kunnen beïnvloeden. In studies met huid epitheel bleken deze groeifactoren de wondheling inderdaad te stimuleren. Een overmatige aanwezigheid van deze groeifactoren kan zelfs fysiologische processen dereguleren en tumorvorming bevorderen. Er is echter weinig bekend over effecten en functies van groeifactoren in de biologie van blaas epitheel. In dit proefschrift zijn 1) effecten van diverse groeifactoren op muizeblaas epitheel in vitro bestudeerd, 2) expressie van groeifactoren en hun receptoren op RNA en eiwit niveau onderzocht gedurende urotheel wondheling in vivo, en 3) is een groeistimulerende factor uit een urotheliale tumorcellijn gedeeltelijk gekarakteriseerd.

In hoofdstukken 2 tot en met 5 is gekeken naar de aanwezigheid en functionele betekenis van groeifactoren vorr proliferatie, differentiatie, migratie, en wondgenezing van "normaal" urotheel. Hoofdstuk 2 beschrijft een in vitro model waarin het muizeblaas epitheel sterk lijkt op muize urotheel in vivo. Hierbij wordt een primaire culture van muizeblaas epitheel op poreuze (Cyclopore) membranen gebruikt. Diverse parameters voor proliferatie, differentiatie, expansie, en apoptose konden tegelijkertijd op een culture worden gemeten. Gebruikmakend van dit model is daarna bestudeerd hoe groeifactoren deze parameters konden beïnvloeden. Dit is gedaan voor twee modellen: een confluente culture dat intact muizeblaas epitheel vertegenwoordigd, en een niet-

confluente, groeiende culture dat urotheel tijdens wondheling vertegenwoordigd, zoals beschreven in hoofdstuk 3. In beide modellen waren de effecten min of meer hetzelfde. FGF-1 en EGF stimuleerden zowel proliferatie als migratie, terwijl TGFß$_1$ de terminale differentiatie en de apoptose induceerde. FGF-2 en groeifactor uit bloedplaatjes AA (PDGF-AA) hadden geen meetbaar effect, terwijl PDGF-BB alleen de migratie in geringe mate stimuleerde. Hoofdstuk 4 beschrijft de RNA en eiwit expressie patronen van groeifactoren en groeifactor receptoren gedurende de wondheling van beschadigd muizeblaas epitheel. Deze expressie patronen zijn vergeleken met de veranderingen in de morfologie en de proliferatie tijdens het helingsproces. De verwondingen bleven beperkt tot met name de urotheel cellaag. Op de expressie van TGFß$_1$ en TGFß type II receptor in de spier na bleven de mate en verdeling van de groeifactor en receptor expressie in de stromale en de spierlaag onveranderd. Dit suggereert dat het urotheel voor zijn eigen regeneratie voornamelijk afhankelijk is van zijn eigen groeifactoren. De mRNA expressie van FGF-1, IGF-II, en de type I IGF receptor was het hoogst gedurende de maximale migratie en de hoogste proliferatie aktiviteit in het urotheel. De mRNA en eiwit expressie van TGFß$_1$ en de TGFß type II receptor was met name hoog gedurende de migratie en de terminale differentiatie van het urotheel. Deze resultaten zijn in overeenstemming met de effecten van groei-factoren op urotheel cellen in vitro (hoofdstukken 3 en 5). Ze duiden erop dat FGF-1, IGF-II, en TGFß$_1$ een belangrijkere rol in de fysiologie van muizeblaas epitheel spelen dan FGF-2 of PDGF.

In hoofdstukken 5 en 6 is gekeken of normale en niet-normale urotheel cellen verschillend reageren op groeifactoren. Daarvoor zijn drie verschillende muize urotheel cellijnen opgezet en gekarakteriseerd (hoofdstuk 5). Iedere cellijn leek een stadium uit de transitionele cel carcinogenese te vertegenwoordigen: cellijn g/G lijkt het meest op normale urotheel cellen; NUC-1 is een weinig gedifferentieerde, tumorigene cellijn; en NUC-5 vertegenwoordigd een tussenstadium. Epidermale groeifactoren (EGF, TGFα) en fibroblast groeifactoren (FGF-1, FGF-2) stimuleerden de proliferatie van g/G en NUC-5. Transformerende groeifactor beta 1 (TGFß$_1$) inhibeerde de proliferatie alleen van op normaal urotheel gelijkende g/G cellen, wat een gebruikelijke bevinding is voor de meeste epitheliale cellen. De proliferatie van NUC-1 werd onder de gegeven experimentele omstandigheden behalve door insuline-achtige groeifactoren (IGF-I, IGF-II) nauwelijks door groeifactoren beinvloed. Zo'n autonome groei wordt ook bij andere aggressieve carcinoma cellen gevonden. De respons van urotheel cellen kan dus afhankelijk zijn van het stadium in de transitionele cel

carcinogenese.
In overgangscel carcinomen (OCC) wordt frequent een hyperplasie van het aanliggende urotheel aangetroffen. Eerdere studies aan onder andere coloncarcinomen suggereren dat deze hyperplasie veroorzaakt wordt door groeifactoren afkomstig van nabij gelegen tumorcellen. In hoofdstuk 6 is beschreven dat wanneer NUC-1 cellen getransplanteerd zijn in de blaaswand van syngene muizen, het over de tumorcellen liggende urotheel inderdaad hyperplastisch wordt. Geconditioneerd medium van NUC-1 cellen bleek in vitro de proliferatie van g/G cellen sterk te stimuleren. Door middel van een serie in vitro studies (chemisch-fysische analyse; bioassays; RNA analyse) is de proliferatie-inducerende factor gedeeltelijk gekarakteriseerd. Deze factor blijkt FGF-achtige kenmerken te hebben, maar lijkt niet een van de bekende FGFs te zijn. De (verhoogde) productie van FGF eiwitten (waaronder FGF-1 en FGF-2) is ook in andere studies gevonden in OCC van muis en mens. Deze FGFs stimuleren de proliferatie van urotheel cellijnen.

Toekomstige studies moeten uitwijzen of en welke rol deze groeifactoren spelen in het humane urineblaas epitheel. Andere studies hebben laten zien dat humane OCC afwijkingen vertonen in eiwit of RNA expressie van sommige groeifactoren of groeifactor receptoren. Er is niet bekend of deze groeifactoren groei-stimulerende of -remmende effecten hebben op de OCC cellen zelf of op omringende (urotheel) cellen. Dit zou verder onderzocht kunnen worden gebruik makend van het in vitro model uit hoofdstuk 2 voor primaire cultures van humane OCC cellen. Tenslotte kunnen dergelijke studies aangeven of groeifactor derivaten gebruikt zouden kunnen worden in de therapie van OCC of chronische vormen van blaasontsteking (interstitiële cystitis).

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LIST OF PUBLICATIONS

1) HM Wortelboer, CA de Kruif, WI de Boer, AAJ van Iersel, HE Falke, and BJ Blaauboer. Induction and activity of several isoenzymes of cytochrome P450 in primary cultures of rat hepatocytes, in comparison with in vivo data. Mol Toxicol 1: 373-381, 1989.

2) WI de Boer, JMJ Rebel, JA Foekens, M Vermey, and TH van der Kwast. Characterization of mouse urothelial cell lines in different phases of transitional-cell carcinogenesis. Int J Cancer 54: 1022-1027, 1993.

3) WI de Boer, JMJ Rebel, M Vermey, CDEM Thijssen, and TH van der Kwast. Multiparameter analysis of primary cultures grown on Cyclopore membranes. J Histochem Cytochem 42: 277-282, 1994.

4) WI de Boer, JMJ Rebel, M Vermey, AAW de Jong, and TH van der Kwast. Characteri-zation of distinct functions of growth factors in murine transitional epithelial cells in primary culture. Exp Cell Res 214: 510-518, 1994.

5) WI de Boer, AGP Schuller, M Vermey, TH van der Kwast. Expression of growth factors and receptors during specific phases in regenerating urothelium after acute injury. Am J Pathol 145: 1199-1207, 1994.

6) WI de Boer, JMJ Rebel, CDEM Thijssen, M Vermey, AJM van den Eijnden-van Raaij, and TH van der Kwast. Hyperplasia of epithelium adjacent to carcinoma cells is regulated by growth factors through paracrine pathways. Virchows Arch, in press.

7) JMJ Rebel, WI de Boer, CDEM Thijssen, M Vermey, EC Zwarthoff, and TH van der Kwast. An in vitro model of intra-epithelial expansion of transformed urothelial cells. Int J Cancer 54: 846-850, 1993.

8) AGP Schuller, DJ Lindenbergh-Kortleve, WI de Boer, EC Zwarthoff, and SLS Drop. Localization of the epitope of a monoclonal antibody against human insulin-like growth factor binding protein-1, functionally interfering with insulin-like growth factor binding. Growth Regulation 3: 32-34, 1993.

9) JM Rebel, WI de Boer, CD Thijssen, M Vermey, EC Zwarthoff, TH van der Kwast. An in vitro model of urothelial regeneration: effects of growth factors and extracellular matrix proteins. J Pathol 173: 283-291, 1994.

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