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Characterization of Distinct Functions for Growth Factors in Murine Transitional Epithelial Cells in Primary Organotypic Culture

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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, TGFβ, FGF-1, FGF-2, PDGF-AA, and PDGF-BB on mouse urothelium in organoid-like primary cultures. Confluent and nonconfluent 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 nonconfluent cultures. TGFβ inhibited proliferation, caused a decrease in the number of cell layers, and blocked expansion. Moreover, TGFβ induced the terminal differentiation and apoptosis of urothelial cells. In nonconfluent 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 TGFβ promotes the development of a normal, differentiated transitional epithelium. © 1994 Academic Press, Inc.

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 β (TGFβ) 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 β receptor expression in skin and renal epithelial cells *in vivo* only under specific conditions. TGFβ 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 TGFβ 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, TGFβ, FGF-1, FGF-2, and PDGF-AA modulated the proliferation of nontumorigenic 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, nonconfluent, 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

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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) TGFβ induces terminal differentiation and apoptosis of urothelium.

MATERIALS AND METHODS

Chemicals. Cell culture media were obtained from Flow Laboratories (Zwanenburg, NL), and media supplements were from Sigma (St. Louis, MO). Porcine insulin, mouse EGF, and human TGFβ_1 were purchased from Sigma. 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, NY). 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 was 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. After being 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, supplemented with 10% heat-inactivated fetal calf serum (FCS), 5 μg/ml insulin and transferrin, 50 n*M* hydrocortisone, 5 ng/ml selenite, 10 μ*M* 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% of the maximal outgrowth area, or to confluency.

Serum-free medium experiments. Experiments with growth factors were performed in serum-free medium as described previously [16]. This medium is similar to the routine medium without FCS, but supplemented with 0.1% bovine serum albumin, 4 μ*M* spermine, 4 μ*M* spermidin, 0.1 m*M* ethanolamine, and 1 μ*M* 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 (nonconfluent 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 h with BrdU (see below). Subsequently, cells were incubated during 6 days with 0.5 ng/ml TGFβ_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 the 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 labeling was done as described previously [15]. In short, upon termination of the culture, cells were first incubated with 40 μg/ml BrdU in routine medium for 2 h and subsequently in fresh standard medium containing 20 μCi [^{3}H]thymidine/ml without Ham's F10 and FCS for another 2 h. After the cells were rinsed with non-labeled thymidine in phosphate-buffered saline (PBS), pH 7.2, cultures were first used for immunohistochemistry. Finally, the incorporated radioactivity was counted in Ultima Gold scintillation liquid using an α,β-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 labeling index (L.I.). The L.I. is defined as the relative number of BrdU-positive nuclei in 12 prefixed areas of 0.15 mm^2 per culture.

Pulse-chase labeling: in some experiments cells were labeled for 8 h with 40 μg/ml BrdU in serum-free medium with the indicated growth factor. The label was chased by an excess of nonlabeled 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]. First, 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 (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 1000 nuclei per culture were 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 lead nitrate-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 $\pm$ the standard deviation. The significance of data was determined using the Student's *t* test.

RESULTS

Model Mimicking Intact Bladder Mucosa

Primary cultures of mouse urothelium were grown to confluency under routine culture conditions. At confluency, cultures were treated for 4 days with growth factors in serum-free medium. Table 1 presents the quantitative data of the parameters for growth (number of nuclei), proliferation (labeling 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 increase of cell layers from two to three up to six layers as confirmed by TEM (Fig. 1). The area of cells which stained for cytokeratin 18, which is consid-

TABLE 1

Effects of Growth Factors on Confluent Primary Urothelial Cultures

Growth factor	Nuclei	L.I.	[^{3}H]THY	RGE53$^+$ area	Cell layers
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
TGFβ (0.5)	124 ± 25	0.1 ± 0.1	917 ± 749	85.3 ± 4.8	1–2
TGFβ (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

Note. 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 labeling index (L.I.), the incorporation of [^{3}H]thymidine (in dpm), the cytokeratin 18-positive area (RGE53$^+$) related to the total growth area (460 mm^2), and the number of cell layers. The TGFβ_1 concentrations are 0.5 or 2.0 ng/ml (indicated in parentheses).

ered 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 (Fig. 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 TGFβ inhibited the proliferation (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 (Fig. 1). The cytokeratin 18-positive area in TGFβ-treated cultures was larger compared to serum-free cultures. The superficial cells were all large with a regular polygonal morphology resembling umbrella cells (Fig. 2). These observations suggest that TGFβ 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 labeling indices were similar to that of cultures treated with serum-free medium alone.

To confirm that TGFβ 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 labeled with BrdU (see Materials and Methods). The cultures were subsequently treated with either TGFβ or serum-free medium alone. Cross sections demonstrated that the number of cell layers in the TGFβ-treated cultures was diminished to one or two (Figs. 3A and 4), while serum-free medium treated cultures were still stratified with at least three cell layers (Figs. 3B and 4). Compared with non-TGFβ-treated cultures, the number of non-BrdU-labeled nuclei was decreased in all cell layers in TGFβ-treated cultures, while the number of BrdU-labeled nuclei remained unchanged (Fig. 4). Finally, the cytokeratin 18-positive area in TGFβ-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 TGFβ-treated cultures. The data given in Fig. 4 show that BrdU was incorporated in the originally basally located cell layers. So, TGFβ-mediated loss of cells occurs from the superficially located cell layers. 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 TGFβ treatment can be regarded as a phenomenon of terminal differentiation.

Furthermore, TGFβ has been reported to induce apoptosis in uterine epithelium [23], hepatocytes [24], and gastric carcinoma cells [25]. In the above-described model for terminal differentiation induction, we examined whether exposure to TGFβ 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 TGFβ. The ratio of apoptotic bodies versus normal appearing cells was 1 of 50 nuclei in cultures treated for 6 days with TGFβ versus 1 of 1000 nuclei in cultures treated for 6 days with serum-free medium alone.

Model Mimicking Regenerating Bladder Mucosa

Nonconfluent 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 Fig. 5A. Treat-

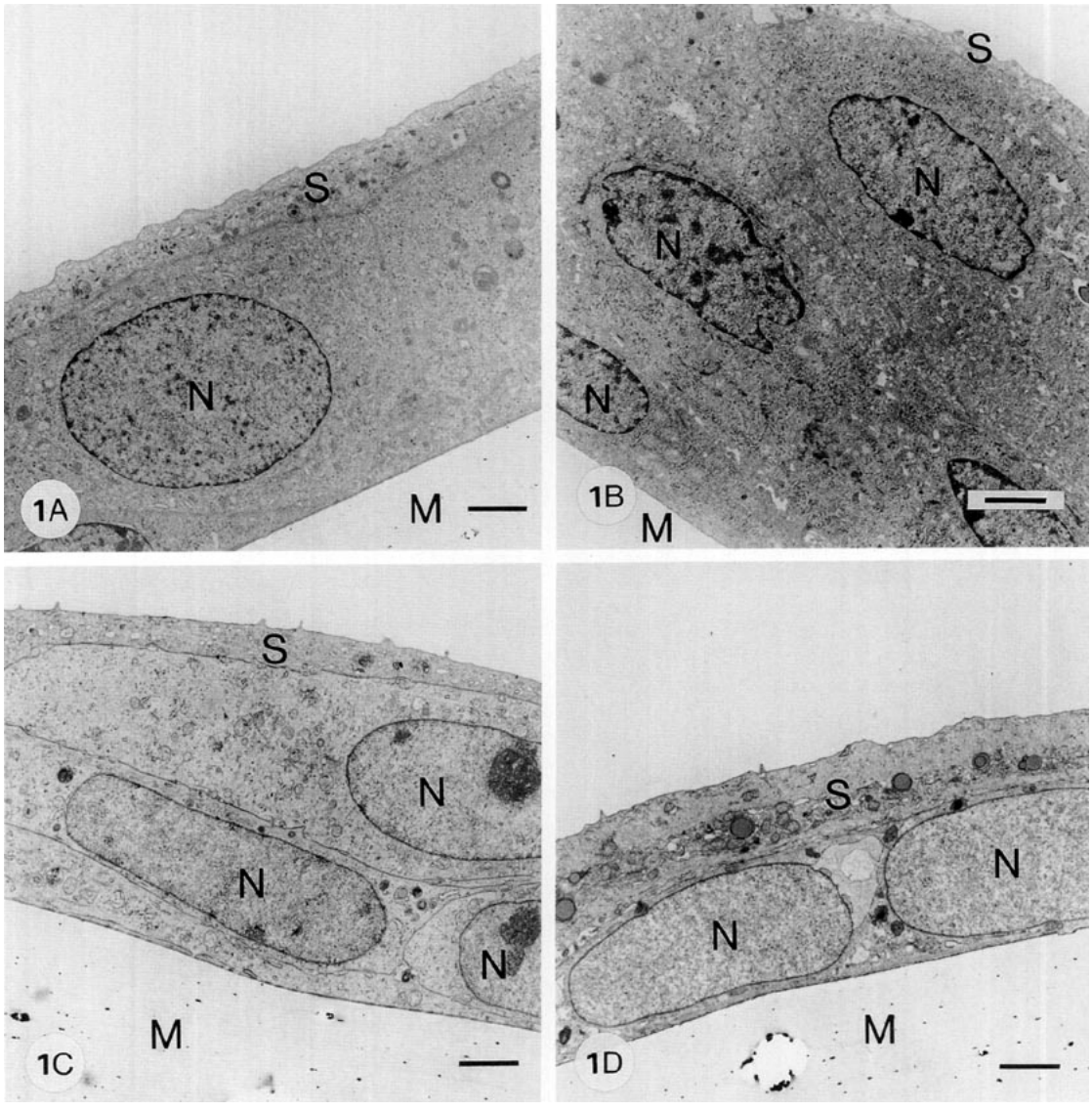

FIG. 1. Transmission electron micrographs of confluent urothelial cultures treated with different growth factors during 4 days. Cultures were treated with serum-free medium alone (A), EGF (B), FGF-1 (C), or 0.5 ng/ml TGFβ_1 (D). N, nucleus; M, membrane; S, superficial cell. Original magnification: 3000×; bar, 2.5 μm.

ment with 0.5 ng/ml TGFβ 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 TGFβ the number of nuclei and the thymidine uptake decreased, but the cytokeratin 18 expression and the number of umbrella cells increased. 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 days with or without PDGF-AA, PDGF-BB, or FGF-2 in serum-free medium. Figure 5B shows the effect of the PDGFs 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

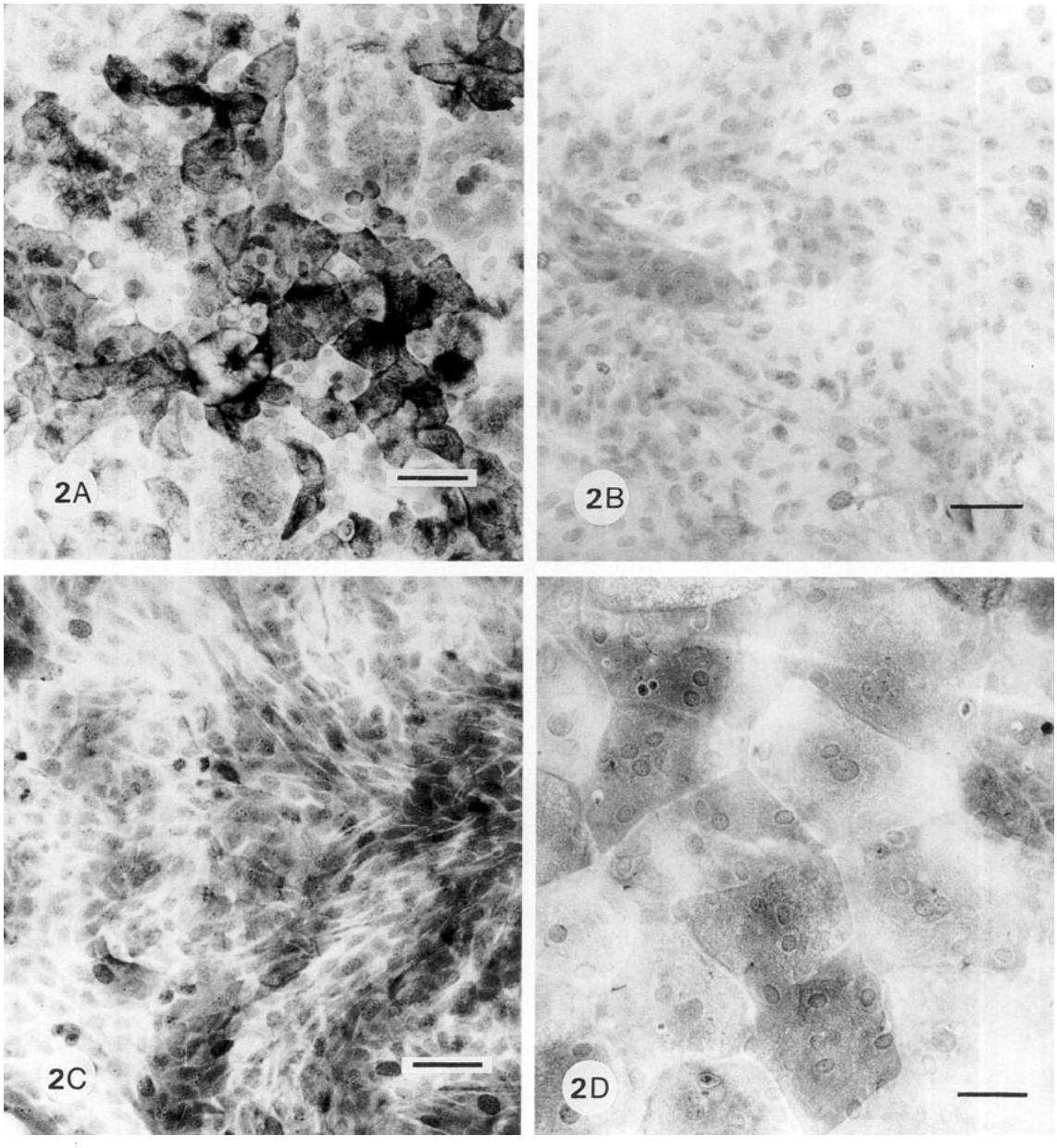

FIG. 2. Micrographs of cytokeratin 18-positive, superficial urothelial cells in confluent cultures treated with different growth factors during 4 days. Cultures were counterstained with hematoxylin. Note the differences in morphology of the superficial cells. Cultures were treated with serum-free medium alone (A), EGF (B), FGF-1 (C), or 0.5 ng/ml TGFβ_1 (D). Original magnification: 200×; bar, 50 μm.

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 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, TGFβ (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

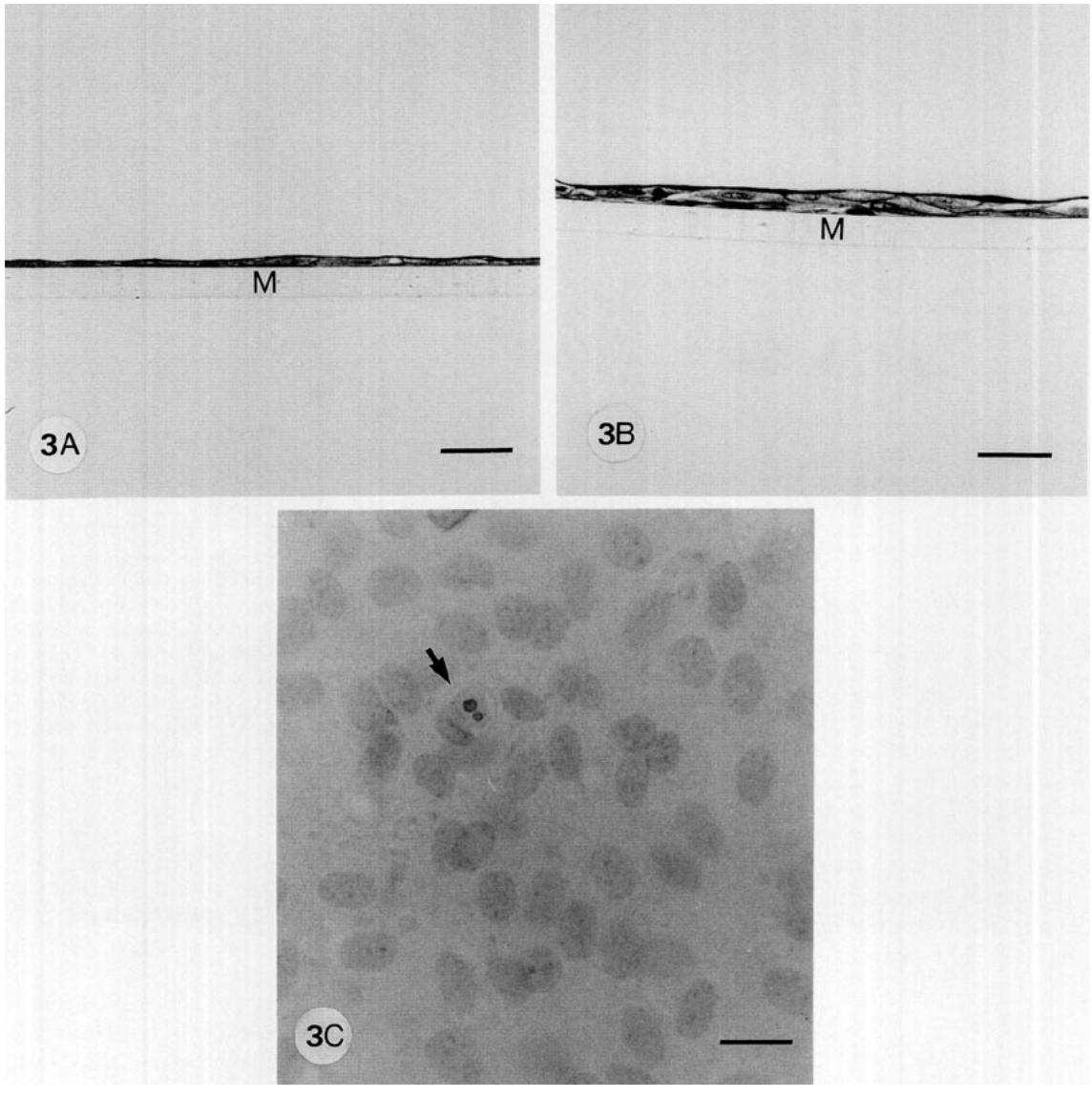

FIG. 3. Induction of terminal differentiation and apoptosis by TGFβ_1. (A, B) Cross sections of paraffin-embedded, hematoxylin-stained, and cytokeratin 18-positive, urothelial cultures treated with either 0.5 ng/ml TGFβ_1 (A) or serum-free medium alone (B) during 6 days after pretreatment with EGF. The cytokeratin 18-positive cells are darkly stained. M, membrane. Original magnification: 400×; bar, 25 μm. (C) Micrograph of Feulgen- plus BrdU-stained urothelial cultures treated with TGFβ_1. The arrow indicates an apoptotic, BrdU-stained body. Original magnification: 500×; bar, 20 μm.

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 (Fig. 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 for publication]. 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 reepithelialization 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.

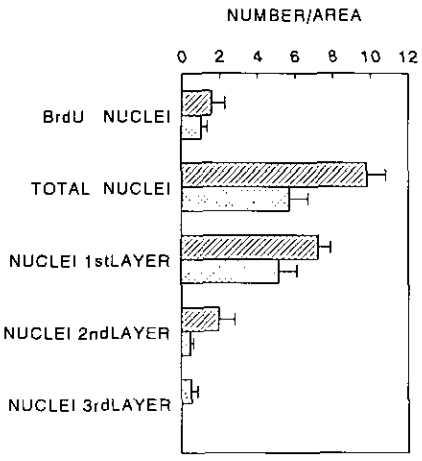

FIG. 4. TGFβ_1 effect on differentiation of confluent urothelial cultures. The number of nuclei counted on cross sections of cultures treated with TGFβ_1 (0.5 ng/ml) (stippled bars) or without TGFβ_1 (hatched bars) is given on the *X* axis. The number of nuclei was counted per cell layer on half of a cross-sectioned membrane or, as with BrdU-labeled nuclei, is given for all cell layers together per half of a cross-sectioned membrane. Data are given ± standard deviation ($n = 6$).

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

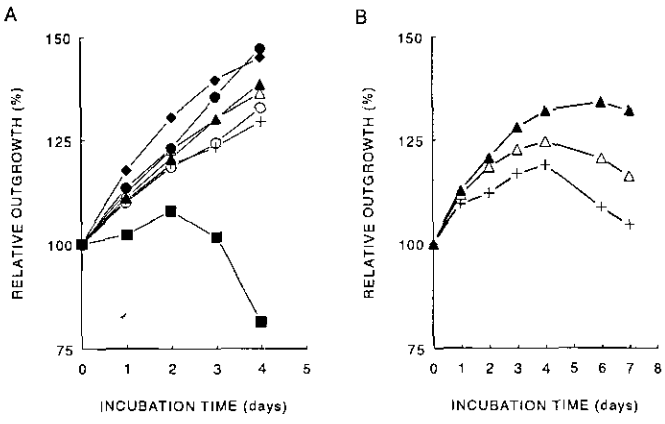

FIG. 5. Effect of growth factors on the increase in the outgrowth of nonconfluent urothelial cultures. When the cultures covered 55 to 65% of the maximal outgrowth area, the incubation started (Day 0); this outgrowth area was set at 100%. The *X* axis indicates the incubation time in days, the *Y* axis the relative increase in outgrowth. (A, B) Cultures with (A) or without (B) the original explant present during incubation. TGFβ_1 concentration was 0.5 ng/ml. (+) = serum-free alone; (●) EGF; (♦) FGF-1; (○) FGF-2; (△) PDGF-AA; (▲) PDGF-BB; (■) TGFβ_1.

TABLE 2

Effects of Growth Factors on Nonconfluent 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		
TGFβ	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		

Note. Effect of growth factors on proliferation and differentiation of nonconfluent 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 labeling index (see legend of Table 1) at the edge of the outgrowth (top row) and the number at the central part of the outgrowth (bottom 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 TGFβ_1 concentration was 0.5 ng/ml. N.D., not determined.

slight induction of urothelial expansion by PDGF-BB and not PDGF-AA, suggesting a functional difference between these two PDGFs in urothelium, although, the biological role of PDGF on epithelia *in vivo* is not yet clear.

We failed to observe an effect of TGFβ 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-day pretreatment with 2–20 ng/ml TGFβ_1. Upon a 3- to 5-day treatment of confluent cultures with 2 or 4 ng TGFβ_1/ml serum-free medium the outgrowth area decreased due to terminal differentiation. After a 2-day treatment with 0.5 ng TGFβ_1/ml serum-free medium, we also noted a decrease in the outgrowth area of nonconfluent cultures. According to Nickoloff *et al.* [35] only undifferentiated cells are able to migrate. This may explain the lack of migration in response to TGFβ_1 in our experiments since TGFβ_1 induced terminal differentiation in our cultures. Hebda [19] observed that TGFβ induced the migration of keratinocytes in the first 3 days of a skin explant culture. In these experiments TGFβ might have affected the migration of only basal (undifferentiated) cells.

Differentiation

Data from this study demonstrated that TGFβ both inhibits the proliferation and induces the terminal dif-

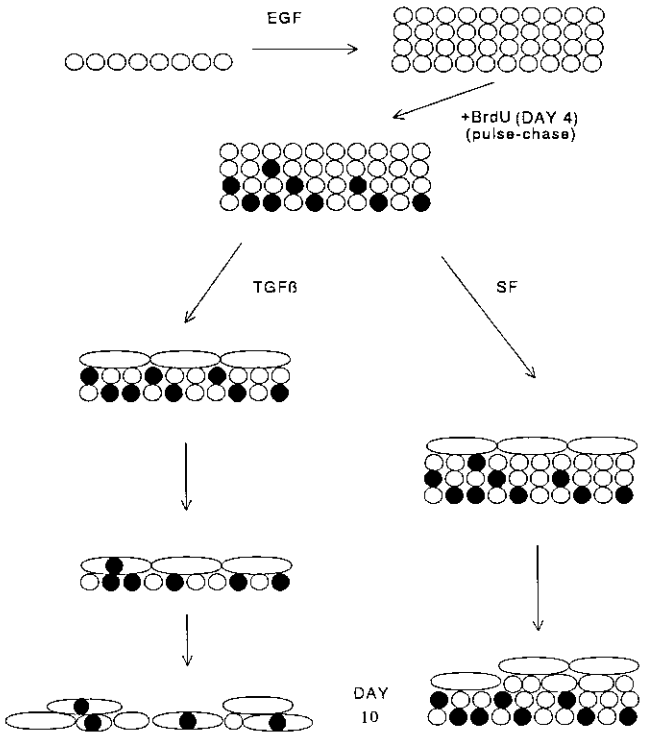

FIG. 6. Schematic diagram depicting the subsequent action of growth factors on proliferation and differentiation of transitional epithelial cells, resulting from the terminal differentiation induction experiments. Circles, ovals, and flattened ovals represent undifferentiated cells, intermediate differentiated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

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

The data of the present study also demonstrate that TGFβ induced apoptosis in transitional epithelial cells. This finding is in line with observations of epithelial cells from uterus [23], liver [24], and a gastric carcinoma [25]. Our results are consistent with a biological function of TGFβ in transitional cell epithelium *in vivo*. TGFβ is present in the urine and in normal urothelial cells [8, 11, 12], and upon injury, TGFβ is released by blood platelets and activated macrophages. Moreover, both the TGFβ_1 and the TGFβ type II receptor protein expressions are enhanced in differentiating urothelium during wound healing of the mouse bladder [De Boer *et al.*, submitted for publication]. Hence, the physiological maintenance of existing or regenerating transitional epithelium may be regulated by TGFβ 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 TGFβ_1 is present especially during the differentiation phase of the epidermis [37]. TGFβ 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 shows 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 the 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 tumor-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 TGFβ 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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