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 [3H]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.

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 epithelial 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 Fleo 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 mimics 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...
simultaneously 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 Laborato-
ries (Zwanenburg, NL), and media supplements were from Sigma (St.
Louis, MO). Porcine insulin, mouse EGF, and human TGFβ, 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 anti-
body RGE-53 against cytokeratin 18 was obtained from Organon Tek.
nika (Oss, NL), and antibody IB55 against 5-bromo-2'-deoxyuridine
(BrdU) was kindly donated by Prof. Dr. F. C. S. Ramaekers (Univer-
sity of Limburg, Maastricht, NL). Tritiated thymidine was obtained
from Amersham (Aldershot, UK), and Ultima Gold was from
Packard (Groningen, NL).

Primary cell culture. Primary cultures of female C3H/Law mouse
urothelium were obtained as described previously [15]. In short, mouse
urothelium was dissected from the bladder and placed onto a
25-mm 0.45-µm pore Cyclopore culture support (Falcon culture
insert, Becton-Dickinson, Etten Leur, NL) with the submu-
scosa facing the support. After being placed in a 6-well dish, the com-
partments were filled with routine culture medium [18] consisting of
1:1 Ham's F10 and Dulbecco's Modified Eagles Medium, supple-
mented with 10% heat-inactivated fetal calf serum (FCS), 5 µg/ml
insulin and transferrin, 50 nM hydrocortisone, 5 ng/ml sodium, 10
µM Heps, and 100 IU/ml penicillin, and 100 µg/ml streptomycin.
Cultures were incubated at 37°C in a humidified atmosphere of 5% CO2.
Cultures were then grown to 55 to 65% of the maximal out-
growth area, or to confluency.

Serum-free medium experiments. Experiments with growth fac-
tors 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 µM spermine, 4 µM
spermidine, 0.1 mM ethanaminole, and 1 µM putrescin. Cells were
incubated at 37°C in a humidified atmosphere of 5% CO2 for 4 days
(confluent cultures) or the indicated culture time (nonconfluent cul-
tures). Without insulin, the primary cultures failed to grow and died
data not shown).

In experiments on terminal differentiation and apoptosis, con-
fuent cultures were first incubated during 4 days with 20 ng/ml EGF
in serum-free medium and pulse-chased during the final 3 h with
BrdU (see below). Subsequently, cells were incubated during 6 days
with 0.5 ng/ml TGFβ3, 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.

[3H]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 µCi/ml BrdU in routine
medium for 2 h and subsequently in fresh standard medium containing
20 µCi/µl [3H]thymidine/ml without Ham's F10 and FCS for another 2
h. After the cells were rinsed with non-labeled thymidine in phos-
phate-buffered saline (PBS), pH 7.2, cultures were first used for
immunohistochemistry. Finally, the incorporated radioactivity was
counted in Ultima Gold scintillation liquid using a 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 label-
ing index (LI). The LI is defined as the relative number of BrdU-
positive nuclei in 12 prefixed areas of 0.15 mm2 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 eth-
anal, 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 monoclo-
nal 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 dianzoni salt. Fast red violet LB with Naphthol AS MX phos-
phate 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 incorpo-
rated 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 mm2.

Transmission electron microscopy (TEM). Confluent cultures
were rinsed with PBS, pH 7.2, and fixed with 1.5% w/v glutardal-
dehyde in 0.1 M cacodylate buffer, pH 7.4. Next, the cultures were
treated exactly as described [15]: Uranylacetate- and lead nitrate-
contrastted ultrathin sections (0.02 µm) were studied using a trans-
mision 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 micro-
scope, 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 [16]. Cytokeratin-stained areas and outgrowth areas were
expressed as percentage of the maximal outgrowth area (460 mm2).

Statistical analysis. All experiments were done at least three
times in duplicate. 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

Model Mimicking Intact Bladder MucoSu

Primary cultures of mouse urothelium were grown to
confluence under routine culture conditions. At con-
fluence, 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 ex-
pression and number of cell layers). Compared to
serum-free medium alone both EGF and FGF-1 stimu-
lated the growth and proliferation (enhanced number of
nuclei, [3H]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

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Nuclei</th>
<th>L.I.</th>
<th>[(^3)H]THY</th>
<th>RGE53* area</th>
<th>Cell layers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum-free</td>
<td>212 ± 49</td>
<td>1.0 ± 0.7</td>
<td>3411 ± 1785</td>
<td>32.1 ± 24.2</td>
<td>1-3</td>
</tr>
<tr>
<td>EGF</td>
<td>377 ± 59</td>
<td>5.6 ± 3.3</td>
<td>9086 ± 2393</td>
<td>51 ± 7.0</td>
<td>3-6</td>
</tr>
<tr>
<td>FGF-1</td>
<td>356 ± 76</td>
<td>3.6 ± 0.6</td>
<td>7372 ± 1093</td>
<td>72.2 ± 1.3</td>
<td>3-6</td>
</tr>
<tr>
<td>FGF-2</td>
<td>207 ± 57</td>
<td>1.1 ± 1.1</td>
<td>4839 ± 1822</td>
<td>54.2 ± 23.5</td>
<td>1-3</td>
</tr>
<tr>
<td>TGFβ (0.5)</td>
<td>124 ± 25</td>
<td>0.7 ± 0.2</td>
<td>917 ± 749</td>
<td>85.3 ± 4.8</td>
<td>1-2</td>
</tr>
<tr>
<td>TGFβ (2.0)</td>
<td>42 ± 23</td>
<td>0.9 ± 0.9</td>
<td>413 ± 14</td>
<td>73.6 ± 7.2</td>
<td>1-1</td>
</tr>
<tr>
<td>PDGF-AA</td>
<td>221 ± 33</td>
<td>1.4 ± 0.9</td>
<td>7954 ± 1261</td>
<td>33.1 ± 6.2</td>
<td>2-3</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>127 ± 25</td>
<td>1.3 ± 0.6</td>
<td>6332 ± 1533</td>
<td>78.3 ± 6.1</td>
<td>2-3</td>
</tr>
<tr>
<td>Serum</td>
<td>264 ± 73</td>
<td>1.8 ± 1.5</td>
<td>11061 ± 1747</td>
<td>42.7 ± 19.1</td>
<td>2-3</td>
</tr>
</tbody>
</table>

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 mm², 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 mm²), and the number of cell layers. The TGFβ concentrations are 0.5 or 2.0 ng/ml (indicated in parentheses).

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 reduced 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 normal urothelial 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 bladder 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 (D). N, nucleus; M, membrane; S, superficial cell. Original magnification: 3000×; bar, 2.5 μm.

Treatment 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 [3H]thymidine uptake. The FGF-1-dependent increase in [3H]thymidine uptake seemed to be transient, since treatment for 2 days led to a 5 times higher [3H]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 TGFβ1 (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, TGFβ1 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 (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. TGFβ₁ effect on differentiation of confluent urothelial cultures. The number of nuclei counted on cross sections of cultures treated with TGFβ₁ (0.5 ng/ml) (stippled bars) or without TGFβ₁ (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 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 epithelium 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 TGFβ₁. Upon a 3- to 5-day treatment of confluent cultures with 2 or 4 ng TGFβ₁/ml serum-free medium the outgrowth area decreased due to terminal differentiation. After a 2-day treatment with 0.5 ng TGFβ₁/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 TGFβ₁ in our experiments since TGFβ₁ 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.

**TABLE 2**

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Nuclei</th>
<th>L.I.</th>
<th>[³H]THY</th>
<th>RGE53° area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum-free</td>
<td>107 ± 49</td>
<td>2.9 ± 2.7</td>
<td>3485 ± 2029</td>
<td>67.3 ± 15.9</td>
</tr>
<tr>
<td>183 ± 48</td>
<td>3.7 ± 3.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>260 ± 52</td>
<td>5.7 ± 3.3</td>
<td>100948 ± 46746</td>
<td>62.4 ± 13.6</td>
</tr>
<tr>
<td>462 ± 49</td>
<td>8.1 ± 1.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF-1</td>
<td>239 ± 68</td>
<td>2.7 ± 2.0</td>
<td>5634 ± 1490</td>
<td>64.9 ± 8.9</td>
</tr>
<tr>
<td>485 ± 1.14</td>
<td>9.2 ± 2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF-2</td>
<td>116 ± 14</td>
<td>0.9 ± 0.5</td>
<td>1792 ± 11</td>
<td>65.1 ± 10.4</td>
</tr>
<tr>
<td>206 ± 26</td>
<td>1.0 ± 0.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ</td>
<td>57 ± 5</td>
<td>0.0 ± 0.0</td>
<td>194 ± 35</td>
<td>90.5 ± 6.5</td>
</tr>
<tr>
<td>89 ± 34</td>
<td>0.0 ± 0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF-AA</td>
<td>147 ± 40</td>
<td>2.1 ± 2.1</td>
<td>1833 ± 1138</td>
<td>78.7 ± 14.3</td>
</tr>
<tr>
<td>231 ± 28</td>
<td>1.0 ± 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>126 ± 33</td>
<td>2.0 ± 2.0</td>
<td>N.D.</td>
<td>75.2 ± 7.8</td>
</tr>
<tr>
<td>174 ± 18</td>
<td>4.6 ± 2.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. 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 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 [³H]thymidine (in dpm) and the cytokeratin 18-positive area relative to the outgrowth area (RGE53°) are given. The TGFβ₁ concentration was 0.5 ng/ml. N.D., not determined.

Data from this study demonstrated that TGFβ both inhibits the proliferation and induces the terminal dif-
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 TGFβ1 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 TGFβ1 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.

We thank Mr. F. L. van der Panne for photography and Professor F. C. S. Ramaekers (Department of Molecular Cell Biology, University of Limburg, Maastricht, NL) for providing the antibody against BrdU.

REFERENCES