Expression of Transforming Growth Factor \( \beta_1 \) and Its Receptors in Normal Human Urothelium and Human Transitional Cell Carcinomas

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Previous studies indicated that transforming growth factor \( \beta_1 \) (TGF\( \beta_1 \)) is expressed by normal urothelial cells and exerts regulatory autocrine functions in urothelial maintenance and wound healing. However, little is known about the expression patterns of TGF\( \beta_1 \) and its receptors in bladder tumors. Therefore, we studied the protein and mRNA localization of TGF\( \beta_1 \) and TGF\( \beta \) receptor types I and II (TGF\( \beta \)RI and TGF\( \beta \)RII) in normal human urothelium and transitional cell carcinomas (TCCs) of different grades and stages. Expression of TGF\( \beta \) and its receptors was examined by immunocytochemistry and mRNA in situ hybridization in normal urothelium and TCCs using a semiquantitative method. By immunocytochemistry, the expression of TGF\( \beta_1 \) and TGF\( \beta \)RII was higher in superficial and basal cell layers of normal urothelium than in the intermediate layer. A similar localization was seen in superficial TCCs. TGF\( \beta \)RI was mainly present in basal and intermediate cell layers of normal urothelium and superficial TCCs. In contrast, in muscle invasive TCCs, all tumor cells stained intensely for all three proteins. No correlation was found between immunostaining and TCC grade. In situ hybridization pointed out that all cell layers in normal urothelium exhibit similar TGF\( \beta_1 \) mRNA levels. Elevated TGF\( \beta_1 \) mRNA levels were noted in TCCs irrespective of grade or stage. In conclusion, these data indicate that in normal urothelium TGF\( \beta_1 \), TGF\( \beta \)RI, and TGF\( \beta \)RII expression depend on maturation and differentiation. This pattern is particularly lost in muscle invasive TCCs, in which the expression of the three proteins is enhanced. These data suggest autocrine TGF\( \beta_1 \) mechanisms in human TCC cells that may be more pronounced in muscle invasive TCC cells.

Bladder cancer is diagnosed in approximately 50,000 patients per year and results in about 11,000 deaths annually in the United States. The disease accounts for approximately 5% of all human cancers and represents 95% of all urothelial tumours. For clinical purposes, superficial (stage Ta and T1) and muscle invasive (stage T2 to T4) bladder cancers are distinguished. Low-grade, superficial tumours, particularly stage Ta tumours, have a low risk of progression to advanced or metastatic disease. High-grade or muscle-invasive tumours are more likely to disseminate. Many studies investigated the molecular basis for tumour progression, invasion, and subsequent metastasis. Among the growth factors or their receptors that may be implicated in these processes are transforming growth factor-\( \beta_1 \), TGF\( \beta_1 \), and its receptors. Recently, expression studies by Kubota et al and Miyamoto et al suggested that TGF\( \beta_1 \) is involved in the pathogenesis of human bladder cancer.

TGF\( \beta_1 \) is the most commonly studied growth factor within the TGF\( \beta \) family. TGF\( \beta_1 \) is generally a growth inhibitor of epithelial cells by reducing the ability of cells to enter the cell cycle S phase. TGF\( \beta_1 \) affects differentiation, migration, and apoptosis of epithelial cells including normal urothelial cells. TGF\( \beta_1 \) is not only expressed by blood platelets and macrophages but also by urothelial cells and also occurs in the urine of normal individuals. The concentration of TGF\( \beta_1 \) in the urine of patients with transitional cell carcinoma (TCC) does not change but is elevated in case of obstructive uropathy.

A recent report suggested that levels of TGF\( \beta_1 \) mRNA are higher in TCC, especially in less differentiated TCCs, than in normal bladder epithelium as analyzed by reverse transcriptase polymerase chain reaction (RT-PCR). In addition, superficial tumors were reported to have higher levels of TGF\( \beta_1 \) mRNA than muscle-invasive tumors. On the contrary, Kubota et al did not observe a significant correlation between TGF\( \beta_1 \) transcript levels and tumor stage and grade as analyzed by Northern blotting. Opposing all these data, King et al reported that TGF\( \beta_1 \) proteins are absent from both normal bladder and TCCs as analyzed by immunocytochemistry.
Little is known about the localization patterns of TGFβ₁ and the TGFβ receptor types I and II in human TCCs. Moreover, the reported TGFβ₁ protein and mRNA expression data in human TCCs are conflicting. Therefore, we examined the TGFβ₁, TGFβRI, and TGFβRII protein or mRNA localization patterns in human TCC of different grades and stages, as well as in normal urothelium.

**MATERIALS AND METHODS**

**Chemicals**

Polyclonal rabbit anti-human antibodies raised against a synthetic TGFβRI peptide (ranging from amino acids 158-179, carboxyl terminal domain), and against a synthetic TGFβRII peptide (spanning amino acids 550-565, carboxyl terminal domain) and their neutralizing control peptides were purchased from Santa Cruz Biotechnology (Santa Cruz, NM). The secondary antibodies were purchased from Dako (Glostrup, Denmark) and normal human serum from Sanofi Diagnostics Pasteur (Paris, France). The rabbit polyclonal anti-human antibody against TGFβ₁ was donated by Dr W. Boersma (C.D.I., Lelystad, The Netherlands). The chainspecific cytokeratin 19 antibody (RCK 108) was donated by Dr E.C.S. Ramaekers (University of Limburg, Maastricht, The Netherlands).

**Biomaterial**

At the Department of Urology (University Paris XII, Créteil, France), fresh normal and TCC specimens of 64 patients were obtained by transectional resection, radical cystectomy, nephro-ureterectomy, or normal donor tissue. The tissue samples were processed for immunocytochemistry. Paraffin-embedded tissues derived from 56 patients (18.1% women and 81.2% men) aged 34 to 92 years (mean, 63.3 years) were processed for in situ hybridization. The staining intensity was scored as a number between zero and five (0 = absence of staining; 1 = faint staining; 2 = moderate staining; 3 = moderate to strong staining; 4 = strong staining; 5 = very intense staining) as determined independently by two examiners (V.I.; W.I.d.B.). In cases of disagreement, a third examination under a double-headed microscope was carried out. A sample was considered as negative (score: 0) if the immunostaining was the same as the negative control, and very intense (score: 5) if immunostaining was similar to the positive control.

**Immunocytology**

Immunocytochemistry was performed essentially as described.15 Serial frozen tissue sectioning was performed with a control hematoxylin and eosin–stained section at the beginning and the end of 20 subsequent sections to confirm the presence of normal urothelium or TCC. Antigen expression was demonstrated with appropriate dilutions of the primary antibodies in conjugated immunoenzyme assays using a secondary alkaline phosphatase–conjugated swine anti-rabbit or rabbit anti-mouse antibody. The diazoinum salt Fast red violet LB was used as chromogen with Naphtol AS-MX phosphate as coupling agent. Finally, the sections were counterstained with Mayer’s hematoxylin. The antibody RCK 108 served as a positive control, and PBS with bovine serum albumin as a negative control.

The possibility of false-positivity with antibodies raised against TGFβRI and TGFβRII was verified (and found negative) by preabsorption of the first antibodies with their specific neutralizing control peptides as described by the manufacturer. The staining intensity was scored as a number between zero and five (0 = absence of staining; 1 = faint staining; 2 = moderate staining; 3 = moderate to strong staining; 4 = strong staining; 5 = very intense staining) as determined independently by two examiners (V.I.; W.I.d.B.). In cases of disagreement, a third examination under a double-headed microscope was carried out. A sample was considered as negative (score: 0) if the immunostaining was the same as the negative control, and very intense (score: 5) if immunostaining was similar to the positive control.

**In Situ Hybridization**

Serial paraffin-embedded tissue sectioning was performed for in situ hybridization. The presence of normal urothelium or TCC was routinely checked after each 20 subsequent sections. In situ hybridization with specific TGFβ₁ cRNA probes was performed using a nonradioactive method. A 500-bp Smal-BamHI TGFβ₁ cDNA cloned in pBluescript KS was used as a positive control and PBS with bovine serum albumin as a negative control.

<table>
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<th>Immunoassaying Score</th>
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<th>2</th>
<th>3</th>
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<td>4</td>
<td>4</td>
<td>1</td>
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NOTE. Analysis of the urothelial TGFβ₁ mRNA expression patterns in normal bladder or TCC specimens. The distribution of the immunostaining score is given for TCGs with regard to the indicated tumor grade or stage. N = the examined number of the indicated specimens. The scoring analyses are described in the Materials and Methods section.

<table>
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<tr>
<th>TCC</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>pTa+pT1</th>
<th>pT2</th>
<th>pT3+pT4</th>
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<td>3</td>
<td>9</td>
<td>5</td>
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<td>5</td>
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NOTE. Analysis of the bladder specimens with regard to histopathologic grading and staging. Given are the numbers of specimens for each of the normal tissues or TCC with regard to tumor grade or stage (indicated in parentheses) as used for either immunocytochemistry or in situ hybridization.
FIGURE 1. Micrographs from immunostained normal ureter tissues. (A) TGFβ; protein staining in normal ureter urothelium. (B) TGFβ; mRNA localization in normal ureter urothelium. (C) TGFβRII expression on normal ureter urothelium. Paraffin sections for mRNA in situ hybridisation are not counterstained (B). Other photographs are from frozen sections and are counterstained with hematoxylin. L = lumen; S = interstitial tissue. (Original magnification ×200.)

FIGURE 2. Micrographs from immunostained superficial bladder TCC. (A) TGFβ; protein staining; (B) TGFβ; mRNA localization; and (C) TGFβRII expression. Paraffin sections for mRNA in situ hybridization are not counterstained (B). Other photographs are from frozen sections and are counterstained with hematoxylin. L = lumen; S = interstitial tissue. (Original magnification ×200.)
FIGURE 3. Micrographs from immunostained muscle invasive TCC. (A) TGFβ1 protein staining; (B) TGFβ1 mRNA localization; and (C) TGFβRII expression. Paraffin sections for mRNA in situ hybridization are not counterstained (B). Other photographs are from frozen sections and are counterstained with hematoxylin. L = lumen; S = interstitial tissue. (Original magnification ×200.)

(Stratagene, La Jolla, CA) was obtained as described. The TGFβ1 cRNA probes were labeled with digoxigenin as described by the manufacturer (Boehringer, Mannheim, Germany). The in situ hybridization was performed essentially as described. Briefly, after pretreatment of the sections, the hybridization was performed in hybridization buffer containing 50% formamide at 62°C overnight. After subsequent washings and RNase T1 treatment, the digoxigenin-labeled hybrids were detected by immunohistochemistry (Boehringer, Mannheim, Germany) using nitro blue tetrazolium as chromogen and bichollylindolyl phosphate as coupling reagent. The sense probes were included as negative controls, and did not hybridize. The staining intensity was expressed as described for the immunocytochemistry.

FIGURE 4. Analysis of immunostaining scores. The mean of the semiquantitative scores for the TGFβ1 protein (A), TGFβRI (B), and TGFβRII (C) are given for normal urothelium (open bars), superficial (hatched bars), and muscle invasive (closed bars) TCC, respectively. With regard to the epithelial cells, the number of positively stained bladder specimens is given relative to the total number of the respective specimens (normal bladder: n = 19; superficial TCC: n = 14; muscle invasive TCC: n = 23). The immunostaining scores are given separately for the superficial (S), intermediate (I), and basal (B) urothelial cell layers. Note that no discrimination could be made for the immunostaining patterns of the individual cell layers in muscle invasive TCC. The scoring analysis is described in the Materials and Methods section.
RESULTS

Immunocytochemistry

Both superficial and basal cell layers of the normal urothelium displayed a stronger immunostaining for TGFβ1 and TGFβRII than the intermediate cell layers (Figs 1 and 4). In contrast, TGFβRI was mainly expressed in the basal and intermediate cell layers (Fig 4). TGFβ1 and both receptors were only weakly expressed by smooth muscle cells, whereas in stromal cells little immunostaining was detected. Bladder and ureter tissues showed similar expression patterns for all three proteins (see also De Boer et al).8

The distribution patterns of TGFβ1 and TGFβRII in superficial TCCs were similar to those described for normal urothelial tissues as being preferentially present in the superficial and basal cell layers than in intermediate TCC cell layers (Figs 2 and 4). In contrast to normal urothelium, TGFβRI was evenly expressed in all superficial TCC cell layers but with lower intensity compared with normal urothelium (Figure 4). Smooth muscle cells underlying the TCC expressed the three proteins only faintly, and staining of neither stromal nor endothelial cells was noted. Many muscle-invasive TCC displayed a strong expression of TGFβ1 and both receptors in all cell layers (Figs 3 and 4). When compared with normal urothelium or superficial TCCs, smooth muscle cells within a muscle-invasive TCC showed a stronger expression of TGFβ1, whereas the receptor expression did not change. In addition, endothelial cells, which are numerous in muscle invasive TCCs, were intensely stained for TGFβ1 which contrasts endothelial staining in normal bladder or superficial TCC.

In Situ Hybridization

In concordance with the immunostaining patterns, TGFβ1 mRNA was present in both normal urothelium and in TCC. All urothelial cell layers in normal urothelium and superficial TCCs contained similar transcript levels (Table 2). The TGFβ1 mRNA levels in normal urothelium tended to be lower than in TCC. Poorly differentiated TCC showed a more heterogenous expression level (Table 2). TGFβ1 mRNA was not detectable in the muscular cells of the bladder wall of either normal bladder or TCC, and macrophages near TCC cells were faintly stained (data not shown).

DISCUSSION

The presence of TGFβ1 mRNA in human TCCs was shown in a limited number of studies.4,5,17 As yet, the combined localization of TGFβ1 and its receptors in normal human bladder or TCCs has not been documented. The current study shows that human urothelial cells synthesize and express TGFβ1 and TGFβ receptor types I and II. We also showed the distribution pattern of the respective proteins. The current data point to autocrine functions for TGFβ1 not only in normal urothelial cells5,10-12 but also in TCC cells.

Only a few previous studies showed the presence of TGFβ1 mRNA in TCCs of the urinary bladder. Coombs et al17 observed by Northern blot analysis that TGFβ1 mRNA levels were reduced in about 27% of TCC of grade I or II, and in three of five TCC of grade III as compared with normal bladder. No correlation with TCC stage was found. In contrast, Northern blot analysis by Kubota et al18 and RT-PCR analysis by Miyamoto et al19 indicated a trend toward enhanced TGFβ1 mRNA levels in about 60% of superficial grade I or II TCC (stage Ta or T1), and in 13% to 30% of muscle-invasive TCC (stage T2 or higher) of grade 3 as compared with the TGFβ1 mRNA level in T24 cells. This suggests an inverse correlation of TGFβ1 mRNA levels with TCC grade or stage. In these three studies, the remainder of the TCCs exhibited unchanged levels. A major problem with these observations on tissue homogenates is the presence of nontumor tissue, which may contribute to the TGFβ1 mRNA levels. Furthermore, bladder tumor TGFβ1 mRNA levels in the studies by Kubota et al18 were analyzed using TGFβ1 mRNA levels in T24 cells as a reference, whereas Coombs et al17 used normal bladder tissue as a reference.

In addition, no direct visualization of the localization of TGFβ1, or its receptor mRNA or protein, was achieved at the cellular level. Although semiquantitatively examined, the current study indicates that TGFβ1 mRNA levels tend to be enhanced in TCC cells. Stromal cells exhibit much lower levels than TCC cells. On the protein level, we observed an increased presence of TGFβ1 proteins concomitant with an increased expression of TGFβRI and TGFβRII in higher-stage TCC. The current data are in striking contrast to those of King et al,20 who did not observe any TGFβ1 staining in normal bladder or TCCs using a specific antibody against mature, intracellular TGFβ1. This may be due to differences in detection levels of both antibodies.

In basal and superficial cell layers of normal urothelium and superficial TCC, the high levels of TGFβ1 mRNA corresponded with TGFβ1 protein levels. In contrast, in intermediate cell layers the TGFβ1 protein level was lower than the mRNA levels. Apart from differences in posttranscriptional translation, for instance, this may point to an epithelial redistribution of TGFβ1 protein from intermediate epithelial cells toward basal and superficial epithelial cells. The cellular localization pattern of TGFβ1 was found to correspond with its function as a differentiation factor in other epithelial cell types such as keratinocytes.4,18 In regenerating murine urothelium in vivo, the intense expression of TGFβ1 proteins in superficial cells was found to be in agreement with its functional role in maturation.11 This is supported by in vitro experiments showing that TGFβ1 stimulates the maturation of murine urothelium into umbrella cells and also induces apoptosis.10 Furthermore, in vitro studies showed that exogenous TGFβ1 induces extracellular matrix synthesis and secretion by basal epithelial cells.8 Because basal urothelial cells express high levels of both TGFβRI and TGFβRII, the presence of TGFβ1 in basal urothelial cells also may be related to its functional role in the synthesis of extracellular matrix components. In muscle-invasive TCC, a
maturation-associated expression pattern was no longer visible. Because TGFβs may also modify epithelial integrin expression patterns,13,19 a functional role of TGFβ1 activation may be a modulation of matrix deposition and integrin expression resulting in migration of TCC cells.

TGFβ1 also may exert paracrine functions. Whereas in superficial TCCs the TGFβ3 expression profile did not differ from normal urothelium, in muscle-invasive TCCs the expression of TGFβ3 was found to be high in all tumor cells. Being a chemoattractant for macrophages and T lymphocytes, the TCC-derived TGFβ1 may lead to an influx of these cells causing an inflammatory response to the tumor cells. Indeed, we observed an influx of macrophages in the tumor area (data not shown). Future studies should further address this point. In addition, the high TGFβ1 expression and the abundance of microvessels in muscle-invasive TCCs suggest its implication in tumor neovascularization as described in animals.20 Finally, TGFβ1 also can exert a negative autocrine growth regulation on normal urothelial cells.10,12 Alternatively, because our antibody recognizes both active and latent forms of TGFβ1 proteins, we cannot exclude that the observed TGFβ1 staining includes latent intracellular TGFβ1 complexes.

In conclusion, in the current study we showed the localization of TGFβ1 and TGFβ receptor types I and II in normal urothelium of bladder and ureter, superficial TCCs, and muscle-invasive TCCs. The differential distribution patterns did not differ much between normal urothelium and superficial TCCs, whereas in muscle-invasive TCCs, all cells were strongly immunostained for all three proteins. These data support the idea that TGFβ1 has apart from a paracrine also an autocrine, intraepithelial function that may vary from maintaining a steady-state in normal urothelium to supporting invasion of TCC tumor cells. Functional studies await a relevant model for bladder cancer to assess possible TGFβ1 effects.

REFERENCES