Clinical Implications of the Expression of Epidermal Growth Factor Receptors in Human Transitional Cell Carcinoma

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ABSTRACT

To evaluate the distribution and density of epidermal growth factor (EGF) receptors (EGF-Rs) on urothelium, immunohistological studies using a monoclonal antibody to the binding portion of the human EGF-R were performed on frozen specimens of normal urothelium (N = 20), urothelium from patients with nonurothelial urological malignancies (N = 15) and inflammatory diseases (N = 8), low grade superficial transitional cell carcinomas (TCC) (N = 13), high grade superficial or invasive TCC (N = 28), and endoscopically normal appearing urothelium from patients with low grade superficial (N = 5) or high grade (N = 21) TCC elsewhere in the bladder (or ipsilateral renal pelvis/ureter). EGF-Rs are found only on the basal layer of epithelial cells (with scattered representation on intermediate cells) in 95% of normal urothelial specimens and 100% of pathological specimens without urothelial malignancy. Alternatively, 92.3% of specimens of low grade superficial TCC and 100% of high grade TCCs had EGF-Rs richly expressed on the superficial as well as the deeper layers of urothelium. This "malignant" distribution of EGF-Rs was also found on all specimens of endoscopically normal appearing urothelium in patients with TCC elsewhere. The density of EGF-Rs correlated closely with tumor grade on both "premalignant" and frankly neoplastic urothelium. We conclude that the expression of EGF-Rs on urothelium favors the interaction of premalignant and malignant tissue with urinary EGF.

To determine if altering the physicochemical environment of urine could interfere with this interaction, the effects of pH on the binding of and growth responses to EGF were assessed on four human TCC cell lines. Scatchard plots demonstrated that varying pH from 5.0 to 7.5 did not significantly change the total number of receptors, but EGF-R affinity was reduced approximately 20-fold as pH decreased from 7.5 to 5 in each TCC target. Similarly, significant growth stimulation by EGF at pH 7.5 was abrogated at pH 5.0 while growth rates in the absence of EGF remained unchanged at lower pHs. It thus appears that urinary acidification may hold promise in the management and prevention of recurrent bladder cancer.

INTRODUCTION

EGF is a protein mitogen which is excreted in urine in high concentrations in a biologically active form (1–4). This provides it with the opportunity to incubate with normal, premalignant, and malignant transitional epithelium of the bladder for long periods of time. From our preliminary observations, it appears that the distribution of EGF-Rs in urothelium provides premalignant and malignant transitional epithelial cells with much greater access than normal urothelium to urinary EGF (5). Furthermore, at least in vitro, normal human urothelial cells are far more refractory to potential tumorigenic effects of EGF such as growth stimulation (6, 7) and induction of ornithine decarboxylase activity (6) than TCC cells are. Thus, while the evidence is still circumstantial, there appear to be several independent lines of experimentation indicating that urinary EGF may play a role in the development and growth of TCC.

Recently, it has been reported that the amounts of EGF and anti-EGF-R antibody binding to frozen preparations of bladder tumors directly correlated with malignant aggressiveness (8–10). We have used different immunohistological methods and reagents to assess the relative abundance of cell surface EGF-Rs on TCC specimens of varying histological grades and stages to independently confirm these observations and to determine if the abnormal quantities and distribution of EGF-Rs seen in TCC are consistently found in premalignant urothelium.

If urinary EGF indeed influences TCC tumorigenesis, then interfering with the EGF-target cell interaction may also provide an important tool for preventing and/or treating human TCC. Because of the ubiquity of EGF, lack of knowledge of conditions [other than renal failure (11)] which affect its urinary excretion, the widespread distribution of its functional receptor, and the continuous exposure of EGF-Rs on premalignant and cancerous uroepithelium to urinary EGF, for such strategies of interference to have clinical utility they must be tailored towards specific characteristics of the urinary tract. In view of the composition of urine and our ability through well proved means to alter its physicochemical properties, relatively simple approaches may be specifically applicable to the management of bladder cancer. With these thoughts in mind, we assessed the in vitro effects of pH on binding of EGF and the growth response of human TCC cells to EGF.

MATERIALS AND METHODS

Immunohistology. Tissue specimens were obtained at surgery from patients with "normal" bladders (men with benign prostatic hyperplasia and women undergoing bladder neck suspension operations for stress urinary incontinence) and ureters (from living renal transplant donors), or apparently normal bladder/ureteral specimens in individuals with non-TCC urological neoplasms (ureteral segments from patients with renal cell carcinoma, bladder specimens from men undergoing radical prostatectomy for Stage ≤ B adenocarcinoma of the prostate). Nonneoplastic pathological bladder and ureteral specimens were obtained from patients with inflammatory urinary tract diseases including recurrent urinary infections, pyovesica, chronic interstitial cystitis, vesical calciuli, and genitourinary tuberculosis. Low grade (Grade 1) superficial TCC (Stage ≤ T1), high grade (Grades 2–3) superficial (Stage Ta, T1, or TIS) or deeply invasive (Stage ≥ T2) TCC, and "premalignant" urothelium (from endoscopically normal appearing areas in patients with TCC elsewhere in the bladder) were also obtained. Normal and pathological bladder specimens were always obtained from the bladder dome, each lateral wall, and the posterior wall; biopsies of premalignant tissue were also obtained from these areas when feasible. Specimens were hemisectioned with one-half being formalin fixed, paraffin embedded, prepared for routine hematoxylin and eosin staining, and examined by members of The Division of Surgical Pathology, University of Wisconsin School of Medicine. The resulting histological diagnoses are reported here. The other half of each specimen was frozen after removal and prepared and stained with 1 µg/ml antibody 528 (Oncogene Science, Inc., Mineola, NY) to the ligand-binding portion of the human EGF-R (12), as described previously (5). Antibody HB3, a mouse monoclonal IgG2 generated to mouse IgG (gift of Dr. Richard Hong), was used as a negative antibody control in concentrations of 20 µg/ml. Sections of human TCC cell line 647V (13) growing as a s.c. abdominal wall tumor in nude female Fischer rats served as a positive tissue control. Human rhabdomyosarcoma cells, A673, which do not bind...
Table 1  Cell surface staining with antibody 528 to EGF receptor on normal urothelium

<table>
<thead>
<tr>
<th>Layers of urothelium</th>
<th>Superficial</th>
<th>Intermediate</th>
<th>Basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Ureter (renal cell carcinoma in ipsilateral kidney)</td>
<td>0 1+ 3+</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>B. Bladder (localized prostatic carcinoma)</td>
<td>0 0 2+</td>
<td>0 0 2+</td>
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Total 0/11 1/11 1/11* 9/11

Table 2  Cell surface staining with antibody 528 to EGF receptor on urothelium from patients with nonurothelial genitourinary malignancies

<table>
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<th>Intermediate</th>
<th>Basal</th>
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<tbody>
<tr>
<td>A. Ureter</td>
<td>0 0 2+</td>
<td>0 0 2+</td>
<td>0 0 2+</td>
</tr>
<tr>
<td>B. Bladder</td>
<td>0 0 2+</td>
<td>0 0 2+</td>
<td>0 0 2+</td>
</tr>
</tbody>
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Total 0/4 0/4 4/4

Table 3  Cell surface staining with antibody 528 to EGF receptor on urothelium from patients with urinary tract inflammation

<table>
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<th>Basal</th>
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<tbody>
<tr>
<td>A. Ureter</td>
<td>0 2+</td>
<td>0 2+</td>
<td>0 2+</td>
</tr>
<tr>
<td>B. Bladder</td>
<td>0 2+</td>
<td>0 2+</td>
<td>0 2+</td>
</tr>
</tbody>
</table>

Total 0/4 0/4 4/4

* 2+, same intensity of staining appearing in simultaneously stained positive tissue controls (human TCC cells, 647V, growing as s.c. tumors in nude rats); *, >10% and <50% of cells in this layer were positively stained.

Fig. 1. Section of midureter from 70-year-old man who underwent nephroureterectomy for genitourinary tuberculosis. A, immunoperoxidase staining (with hematoxylin counterstain) with antibody 528 to human EGF receptor, x 100. Note intense red-brown staining in basal epithelial layer, indicating abundance of EGF receptors on cells of this layer. B, staining same tissue with antibody HB3 to mouse IgG.
where \( N^* \) and \( A' \) are the number of viable cells on days 7 and 3, respectively (23). Murine EGF was used for each experiment except for 647V in DH-SI where human EGF was used. We have shown previously that these cells are not stimulated to grow by mouse EGF when in DH-S1, while they are stimulated by human EGF (6). All experiments were repeated at least twice and always performed in triplicate or quadruplicate. Standard deviations were calculated and \( t \) tests were performed subsequent to a blocked analysis of variance in each group (blocking for experiment).

RESULTS

EGF-R Distribution and Density on Normal, Premalignant, and Malignant Human Urothelial Tissues. Our original observation that EGF-Rs are restricted to the basal layer or to basal and intermediate cell layers of normal uroepithelium (5) has been confirmed in both the ureter and the bladder (Table 1). In addition, the urothelium in a variety of nonuroepithelial neoplastic and nonneoplastic pathological control specimens has a similar EGF-R distribution (Fig. 1; Tables 2 and 3). In normal and pathological controls, no differences in EGF-R distribution could be seen in any of the 4 areas of bladder sampled (not shown). Conversely, endoscopically and often histologically normal urothelial samples from patients with both low grade and high grade TCC elsewhere in the bladder have EGF-Rs richly expressed on superficial cells (Tables 4 and 5).

As can be seen in Tables 4 and 5 and Figs. 2 and 3 the expression of EGF-Rs was greater on high grade/invasive than on low grade superficial TCC. In addition, histologically normal or mildly dysplastic urothelium from high grade TCC patients had more EGF-Rs on all epithelial cell layers than similar specimens from low grade superficial TCC patients (Figs. 4 and 5; Tables 4 and 5). These findings are summarized in Table 6.

Effects of pH on EGF Binding by TCC Cells. As we have reported previously (6) the numbers and affinities of EGF-Rs varied considerably between TCC cell lines. Acidification significantly reduced binding affinities without significantly altering the number of receptors (Table 7; Fig. 6). Linear binding kinetics was seen for each TCC cell at each pH tested (Fig. 6). pH values outside the ranges reported were not tested because they cannot be consistently maintained in humans (pH < 5) or would represent a prohibitive risk for urinary calculus formation (pH > 7.5).

Growth Stimulation. As reported previously, EGF stimulated growth of all human TCC cell line clones in a dose-dependent fashion at pH 7.5 (6) (Table 8). Reduction of pH values to 7.0 and 6.0 only minimally affected growth rates in the absence of EGF but totally abrogated the growth response to EGF (Table 8). No cell grew sufficiently well at pH < 5 to obtain reliable growth curve data (not shown).

DISCUSSION

Clinical (24-26) and experimental (27, 28) observations of spontaneous and induced TCC indicate that substances excreted...
in normal urine may be important in its development and growth. Because EGF, which is excreted in the urine in high concentrations, is known to effect tumor promotion and growth in certain natural tumor systems (29), and has cocarcinogenic properties (30), it is a likely candidate for playing such a role in TCC. Evidence to support this contention has recently been reported by Yura et al. (31). High levels of EGF-Rs have been found on a variety of human tumors, including squamous cell carcinomas from various sites (32, 33), gynecological tumors (34), and some sarcomas (35). Several researchers have correlated increased EGF-R expression with increased malignant behavior for breast tumors (36–38), and tumors of glial origin (39, 40). Berger et al. (8–10), expanding on prior observations by their group (30), showed that: (a) EGF-R expression and EGF binding correlated directly with increased TCC aggressiveness; (b) at least in one TCC specimen, these EGF-Rs had biological (i.e., autophosphorylation) activity; and (c) they did not appear to represent the v-erb B oncogene product, a truncated form of the EGF-R (using monoclonal antibodies to the extracellular and intracellular human EGF-R domains). Furthermore, in only 1 of 29 specimens could gene amplification explain the increased expression of EGF-Rs on TCC (by Southern blot analysis). Our work expands on these findings, independently confirming with different reagents, methodologies, and means of (semi) quantification that increased staining of cell surface EGF-Rs directly reflects the aggressive behavior of TCCs and that EGF-Rs are abnormally expressed on superficial urothelial cells in both low grade and high grade TCC. [These methodological differences probably explain why we found more superficial low grade tumors to have abnormal EGF-R expression than Neal et al. (10) did.]

Indeed the similarity of our findings with those reported by others in itself is notable, because the antibody we used will not avidly bind to EGF-Rs already occupied by ligand, while the reagent used by Neal et al. (10) recognizes both occupied and unoccupied EGF-Rs. However, occupied EGF-Rs will be internalized within minutes and completely degraded shortly thereafter (41). Thus, although it is conceivable that some of the
EGF AND ITS RECEPTOR IN BLADDER CANCER

Fig. 2. Section of superficial Grade 1 TCC of the bladder in 60-year-old woman stained with antibody 528, × 100. Every malignant cell is positively stained, although heterogeneity in intensity exists. Note that the greatest densities of EGF receptors are on cells near the bladder lumen.

Fig. 3. Section of invasive Grade 2–3 TCC of the bladder in 63-year-old man stained with antibody 528, × 100.

Fig. 4. Section of cytoscopically normal appearing bladder tissue from 62-year-old woman with low grade superficial TCC elsewhere in her bladder stained with antibody 528 (A) or antibody HB3 (B), × 100. Note only mild epithelial atypia (B) and the EGF receptor located on all epithelial cells, particularly those of the superficial layer (A).

Fig. 5. Section of cystoscopically normal and histologically minimally dysplastic urothelium from the bladder of the same patient shown in Fig. 3 (high grade invasive TCC elsewhere in bladder) stained with antibody 528 (A) and antibody HB3 (B), each × 100. Note intensely positive staining for the EGF receptor on cells of every epithelial layer (A).

products of EGF-R degradation have sufficient antigenic integrity to react with anti-EGF-R antibodies, it would seem far more likely that the vast majority of immunologically detectable EGF-Rs, particularly at the cell surface, are unoccupied by ligand regardless of where in the receptor the antigenic epitope lies. However, if both normal and malignant appearing urothelium in patients with TCC have many unoccupied EGF-Rs, is
it because urinary EGF does not reach (or bind with) these cells? This seems unlikely because it is known (at least in animals) that intraluminal EGF reaches and stimulates urothelium (5, 31). A more plausible explanation is that only a relatively small number of the EGF-Rs which these cells do express are occupied at any time. Such findings would be expected if it is remembered that the concentrations of extracellular EGF needed to achieve optimal responses are often well below those needed to saturate receptor sites or induce receptor down regulation (41).

Importantly, the distribution of EGF-Rs which characterizes cancer is routinely found on normal appearing urothelium in almost all TCC patients, with the density of receptor expression reflecting the underlying degree of malignancy elsewhere in the bladder. This observation does not appear to simply be an artifact of bladder instrumentation or rapid urothelial cell turnover, since in a variety of inflammatory nonneoplastic conditions EGF-Rs are not present on all cell layers. The expression of EGF-Rs on superficial cells often precedes the standard histological evidence of a preneoplastic state [e.g., moderate or severe dysplasia (24)] and fits in well with two observations of the clinical behavior of bladder cancer; (a) field change/ferile soil phenomena are largely responsible for the multiple recurrences common to all forms of TCC (24); and (b) while both forms of TCC [low grade superficial versus high grade superficial or invasive] may share common steps in their tumorigenesis, one type of tumor does not frequently convert to the other in subsequent recurrences (42–45) implying that at least some consistent differences [all-be-they quantitative] exist in the biological properties of each type of TCC. Certainly these findings are consistent with the hypothesis that exposure to urinary EGF is important for malignant urothelial development and growth.

However, clinically useful strategies to interfere with the EGF-TCC interaction have not been investigated previously. Toxins or radioisotopes conjugated to ligands or anti-receptor antibodies, unconjugated anti-receptor antibodies, and anti-growth factor antibodies have all been used in experimental and therapeutic models of a variety of malignancies (46–48). Thus far, however, the clinical value of these approaches, particularly with as ubiquitous an antigen as EGF or its receptor, has not been demonstrated. The distribution of EGF-Rs within the urothelium would also mitigate against intravesical administration of such agents for treatment of existing tumors because it is unlikely that: (a) those aimed at EGF could successfully inactivate the torrent of new ligand continually excreted in the urine; and (b) those aimed at the receptor could penetrate sufficiently deeply into tumor masses to reach all cells with EGF-Rs. It is, however, possible that if such agents were given intravesically only after resection of all visible tumors they could cause sufficient destruction of the superficial premalignant urothelium to permit them, upon subsequent administration, to reach (and kill) deeper cells with EGF-Rs.

An alternate approach which we have chosen to investigate is based on our finding that the normal appearing urothelium which is left intact after tumors are removed has EGF-Rs on every epithelial cell, particularly those lining the lumen of the bladder. Steps which alter the environment in which urinary EGF and its urothelial receptor interact, such as those that modify the physicochemical properties of urine, may be readily achieved with considerable safety over long periods of time (49, 50). If these steps successfully interfere with the EGF/EGF-R interaction, they would block any stimulatory effects of urinary EGF on cells which normally would bind it. Furthermore, it has been reported that under extremely acidic conditions EGF rapidly dissociates from its receptor (51, 52). In this light, the advantages of urinary acidification become clear.

If EGF truly plays a significant role in TCC development and if acidification does abrogate the effect of EGF on urothelium in vivo, then one might expect to see a reduced incidence of TCC in individuals with continually acidified urine. Unfortunately, no clinical or epidemiological studies have been carried out to evaluate this [e.g., assessing rates of TCC in uric acid stone formers whose inability to alkalize urine is considered their major metabolic defect (50)]. Similarly, excretions and concentrations of urinary components such as the metalloions magnesium, manganese, and calcium, each of which have described effects on the EGF-R (53), have not been measured in a series of TCC patients.

The explanation(s) for the effect(s) of acidification on EGF binding is not known. Acidification even may (reversibly) alter the structure of EGF and activity, since although EGF is known to maintain its chemical stability when it is acidified to pH 4 and realkalimized, its biological activity has not routinely been assessed until the pH has been elevated to 7.4–7.5 (54). In our study, components in the media other than H+, Cl−, and EGF were not deleted or increased. However, we have not investigated the effect of pH on the availability of other substances to the epithelial cells of the bladder or the (potential) influence of acidification on the cell membrane or other receptors which in turn can interact with EGF-Rs. Indeed, it is possible through such mechanisms, rather than (or in addition to) a direct effect on ligand, receptor or the ligand-receptor complex that acidification may act. Furthermore, since, in vivo, urinary acidification undoubtedly will alter other elements of the electrolyte milieu, additional complexities in determining its mode of action will arise. Whatever the mode(s) of action of acidification, it is of interest to note that urinary alkalinization markedly enhances tumor promotion in chemically initiated rat urinary bladder cancer (55).

There were over 45,000 new patients with bladder cancer in 1988 in the United States (56), two-thirds of whom had superficial tumors (57–59). While many of these tumors do not pose a serious threat to life, they recur quite frequently with standard therapies (42, 43). Furthermore, 10–20% eventually progress to more invasive and even life-threatening malignancies (42,

### Table 6 Comparison of mean staining with antibody 528 to EGF receptor on nonmalignant and malignant transitional epithelium

<table>
<thead>
<tr>
<th></th>
<th>Superficial</th>
<th>Intermediate</th>
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<tbody>
<tr>
<td>Nonmalignant* (N = 43)</td>
<td>0.02</td>
<td>0.05</td>
<td>2.51</td>
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<tr>
<td>Superficial papillary TCC</td>
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<td></td>
</tr>
<tr>
<td>Tumor (N = 13)</td>
<td>1.19</td>
<td>1.19</td>
<td>1.81</td>
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<td>Epithelium (cystoscopically normal) (N = 5)</td>
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<td>1.70</td>
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<td>Invasive or high grade TCC</td>
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<tr>
<td>Tumor (N = 28)</td>
<td>2.77</td>
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<td>2.96</td>
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<tr>
<td>Epithelium (cystoscopically normal) (N = 21)</td>
<td>2.74</td>
<td>2.79</td>
<td>3.14</td>
</tr>
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</table>

* Nonmalignant, all nonuroepithelial cancer specimens from Tables 1, 2, 3.
43). There is thus little question that additional therapeutic and particularly preventative modalities need to be developed in the management of TCC. The use of urinary acidification alone or in conjunction with other steps [e.g., inhibition of polyamine synthesis (60)] might well provide a promising approach to this problem.

ACKNOWLEDGMENTS

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REFERENCES

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