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Epidermal Growth Factor and Its Receptor: Markers of—and Targets for—Chemoprevention of Bladder Cancer

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Abstract Epidermal growth factor (EGF) is excreted in urine in high concentrations in a biologically active form. Several lines of evidence indicate that EGF plays a role in transitional cell carcinoma (TCC) development and growth. These include: (1) EGF in the normal urine of rats promotes chemically initiated TCC; (2) EGF in normal human urine stimulates the clonal growth of human TCC cells *in vitro*; (3) EGF stimulates the *in vitro* growth of human TCC cells, but not normal human urothelial cells; (4) the density and distribution of the EGF receptor (EGF-R) on human urothelial tissues permits significant access of premalignant, dysplastic, and malignant cells to EGF; and (5) the concentration of EGF in the voided urine of patients with TCC is reduced, implying that EGF may be "extracted" from urine by the greater number of EGF-Rs in patients with urothelial malignancy.

Abnormal expression of the urothelial EGF-R and/or altered excretion of EGF may well precede overt evidence of TCC and thus may serve as markers of risk or exposure. Similarly, reversion of EGF-R expression or the return of excreted EGF to normal levels may provide a marker of response for preventive and therapeutic strategies. Interference with the EGF/EGF-R interaction through dietary or pharmacological manipulations of the urine, or via targeting strategies employing intravesical administration of conjugated toxins or isotopes is already being employed in experimental and clinical studies. These approaches offer promising new tools in the detection, monitoring, prevention, and management of early stage bladder cancer.

Key words: bladder cancer, chemoprevention, epidermal growth factor (EGF), EGF-receptor

Growth factors (GFs) are a class of proteins that bind to specific cell surface receptors (GF-Rs), inducing a variety of responses, including mitosis, in susceptible target cells. Abnormal production, expression, and/or function of GFs or GF-Rs can result in unregulated growth, a hallmark of malignant transformation. This section focuses on one such GF that has been linked to human bladder cancer—epidermal growth factor (EGF).

Abbreviations: EGF: Epidermal Growth Factor; EGF-R: Epidermal Growth Factor Receptor; GFs: Growth Factors; GF-Rs: Growth Factor Receptors; TGFα: Transforming Growth Factor alpha; TCC: Transitional Cell Carcinoma; TURBT: Transurethral Resection of Bladder Tumor.

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EGF is an ubiquitous protein mitogen of under 6000 molecular weight [1,2]. It is present in many body fluids and is produced by many normal cells: salivary glands, placenta, duodenum, prostate, distal renal tubule, and platelets. However, its physiologic site(s) of production and function(s) remain(s) unknown [1]. Receptors for EGF (EGF-Rs) are present on the membranes of many epithelial cells, and, to a lesser extent, on some connective tissue cells. The inner portion of the EGF-R is an enzyme which phosphorylates tyrosine residues on protein substrates in the presence of ATP. Phosphorylation alters the activities of the target proteins (often enzymes themselves) which sets off a cascade of molecular events culminating in cell division and other responses [3,4]. Binding of EGF to its receptor stimulates the EGF-R's tyrosine kinase activity and initiates events leading to signal transduction [3,5].

EGF AND BLADDER CANCER

There are several lines of evidence indicating that bladder cancer and the urothelium from which it originates represent one of the best human "systems" in which to look for a central tumorigenic role for a GF, particularly EGF. These include: (1) transitional cell carcinoma (TCC) is a common epithelial tumor that is highly representative of human malignancy [6]; (2) urine is an extremely rich source of biologically active EGF [1,2,7]; (3) bladder epithelium has a slow division and turnover rate [8], indicating that it is normally protected from the full effects of urinary EGF; (4) TCC typically occurs in many separate locations in the urinary tract's lining over the patient's lifetime [9], implying an altered susceptibility to normal urinary mitogens such as EGF throughout the urothelium; (5) TCC has been strongly linked to chemical carcinogenesis, particularly to tumor promoters appearing in urine [10]; (6) EGF is known to have a strong role in tumor promotion and growth in both natural [11] and experimental tumor systems [10]; and (7) repeated sampling of tissues and fluids can be done in bladder cancer patients without exceeding the safety and scope of standard medical care due to the unique accessibility of the bladder and the fluid that bathes it.

Thus, studying the EGF/urothelial interaction is feasible, has great potential to further our understanding of urothelial cancer, and may provide clinically valuable information. The evidence to support the hypothesis that EGF is important in uroepithelial carcinogenesis comes primarily from cultured human cell systems and patients' tissues and fluids; we will summarize it here.

EGF IN URINE

High concentrations of EGF are excreted into human urine, where it is allowed to incubate with normal, premalignant, and malignant cells continuously. Blood, lymph, and interstitial fluid also bathe urothelial cells, but concentrations of EGF in these fluids are so small that they are difficult to quantify [12]. Urinary EGF is biologically active, as confirmed by Kuranami *et al.* [13], who found that EGF in normal human urine stimulates clonal growth of human TCC cells. While the accessibility of urinary EGF to cells within the deeper layers of the urothelium or bladder wall of humans is not known, intraluminal EGF can reach the deepest layers of urothelium in rats where it induces proliferative or preproliferative responses [10, 14]. Indeed, Oyasu and co-workers [10,15] have demonstrated that EGF found in normal rat urine promotes chemically initiated TCC in the heterotopically transplanted rat bladder tumor model. Furthermore, a breakdown of the urothelial barrier has been hypothesized to precede the development of overt malignancy and is undoubtedly common in patients with bladder cancer [16]. Thus it is likely that EGF in urine has an opportunity to contact and influence malignant transitional epithelial cells, both at the luminal surface and some distance below it.

Little is known about fluctuations in urinary EGF other than that its excretion diminishes with advanced age and impaired renal function. EGF in voided urine has been measured by four different groups of investigators. Although Matilla *et al.* [7] found no significant differences in EGF excretion between bladder cancer patients and controls, Kristensen *et al.* [17], Fuse and co-workers [18] and Messing and Murphy-Brooks [19] independently found statistically significant reductions in urinary EGF concentrations of bladder tumor patients (tumors were present in the bladders at the time of sample collection in all patients in the last two studies) as compared with those in age- and sex-matched pathologic and normal controls. The potential significance of reduced urinary EGF concentrations in TCC patients will be discussed subsequently.

EGF-Rs IN UROTHELIUM

Possible mechanisms by which the urothelial target cell could respond abnormally to any EGF within its environment would be if EGF-Rs were expressed abnormally, or their structures were altered so that the EGF-Rs functioned abnormally, or both. Though it is possible that both mechanisms play a role, most investigators using human uroepithelial tissues have concentrated on abnormal expression of normal receptors.

Several laboratories [14,20–25] have independently studied expression of urothelial EGF-Rs

using either immunohistochemical methods with antibodies to various portions of the EGF-R or autoradiography with isotope-labeled ligands. All groups have found higher EGF-R expression in malignant than in normal urothelium [20–25], particularly in high-grade/invasive tumors [22,23,25]. Limas has shown that the extracellular portion of the EGF-R, a glycoprotein, shares some of the same carbohydrate residues as those found in A, B, H blood group substances. This fact must be remembered in selecting the appropriate anti-EGF-R antibody, because blood group antigens are often deleted on aggressive tumors [24]. However, despite this and other technical concerns, it is highly unlikely that this observation can be explained away as artifactual considering the similarity of findings using various reagents and methodologies. Whereas it is not yet certain that these receptors are functional in humans, receptor tyrosine kinase activity in at least one patient's tumor was intact [20]. Although many more samples will have to be studied to determine if EGF-R density is truly an independent predictor of tumor behavior, preliminary retrospective analysis indicates that tumors with greater amounts of EGF-Rs have a poorer prognosis [23]. With these findings in mind, it is noteworthy that the gene for the human EGF-R is located on chromosome 7 [26], and that hyperploidy of chromosome 7 is one of the most common cytogenetic findings associated with aggressive TCC [27].

Perhaps more intriguing is that the distribution of EGF-Rs in urothelium may provide an insight into the multifocal and recurrent nature of TCC [6]. This problem has been approached by two groups using different methodologies. Employing immunohistochemistry with an antibody to the ligand-binding portion of the EGF-R, Messing [14,22] found that cells expressing detectable amounts of the EGF-R are strictly confined to the basal layer of normal urothelium and urothelium from a variety of pathological states other than uroepithelial neoplasia. However, in malignant and dysplastic urothelium, EGF-Rs are equally expressed on cells of all urothelial layers, including those directly in contact with urine (*i.e.*, superficial cells). Such altered expression of EGF-Rs, particularly if these receptors are functional, would provide the premalignant and malignant urothelium unusual access to this potent mitogen and tumor promoter.

Looking at the role of tumor implantation after iatrogenic manipulation, See and colleagues [28,29] demonstrated that increased amounts of transforming growth factor alpha (TGFα) and of EGF appear in urine of rats and of humans, respectively, in direct correlation to the degree of trauma. Both TGFα and EGF bind to the EGF-R [30,31] and may stimulate a proliferative response in premalignant or malignant urothelial cells expressing EGF-Rs, including those dislodged from the epithelial surface by iatrogenic or voiding trauma. Both observations could help explain the multiple recurrences that many bladder tumor patients experience.

However, if EGF were to play a truly significant role in TCC tumorigenesis, these alterations in EGF-R distribution should actually precede histologic evidence of neoplasia or preneoplasia and occur in normal-appearing areas of urothelium in patients with TCC tumor diatheses. Such appears to be the case. Normal-appearing tissue (endoscopic and histologic) biopsied from areas geographically remote from obvious tumors have displayed the "malignant" urothelial EGF-R distribution—equal expression on cells of the superficial and intermediate layers as well as on those of the basal layer—in almost all bladder cancer patients examined [22]. These important findings have recently been independently confirmed by Rao *et al.* [25] using immunofluorescence image analysis of touch preparations of cancers and urothelia adjacent to, and distant from, tumors in patients with TCC. Whether such abnormal EGF-R expression is primarily a reflection of impaired maturation or is a more fundamental part of the TCC carcinogenic process is unknown. However, the absence of this distribution in non-neoplastic states of heightened urothelial turnover, such as acute and chronic inflammation, would favor the latter hypothesis [22]. Also unknown is whether this distribution or the density of receptors changes with response to treatment, with progression and/or recurrence, or with failure to recur. However, abnormally expressed receptors binding intraluminal EGF could also explain the diminished amounts of EGF in the voided urine of patients with bladder cancer [17–19]. This concept is

supported by the work of Fuse *et al.* [18] who found that voided urinary EGF concentrations approached normal levels within three to six months following complete transurethral resection of bladder tumors (TURBT) in 12 patients.

On the other hand, this observation is puzzling, because the abnormal EGF-R distribution is often found on tissues that bear none of the histologic features typical of preneoplasia such as hyperplasia or dysplasia [6,22,25]. This may occur because poorly understood barriers may prevent luminal EGF from reaching the cells capable of binding it (although this is unlikely—at least in animals [10,14]) or because some urothelial cells which come in contact with and can bind EGF are not capable of being stimulated by it—at least in a manner germane to proliferation. This hypothesis is supported by *in vitro* findings in which both malignant and normal urothelial cells bind EGF with similar affinities and numbers of receptors; however, only transformed cells are stimulated by this ligand to divide [22,32]. Thus, the abnormal expression of EGF-Rs in urothelium probably represents only one of the many steps in which a urothelial cell becomes responsive to EGF.

In vitro systems have provided excellent models to elucidate the steps occurring after EGF is bound that permit malignant urothelial cells to proliferate while untransformed cells remain unresponsive to EGF although they can bind it. Messing and Reznikoff [5,33] have examined receptor internalization because once EGF-Rs which have bound EGF leave their membrane location, they rapidly lose their capacity to initiate mitogenic responses. Using cultures of human urothelial cells immortalized and malignantly transformed *in vitro*, they found that urothelial cells which bind, but do not respond to EGF, internalize the ligand-receptor complex significantly more rapidly than those transformed urothelial cells which are stimulated by EGF to proliferate [33]. Failure to rapidly internalize the EGF/EGF-R complex could leave the inner portion of the EGF-R—the tyrosine kinase domain—in the "on" configuration longer, resulting in prolonged signalling, perhaps leading to the observed differences in responsiveness to EGF. Employing a different model, Theodorescu, Kerbel and Bruce [34] have demonstrated that transfection of indolent human TCC cells with the normal H-*ras* gene will increase not only expression of EGF-Rs but the proliferative response to EGF as well.

CLINICAL IMPLICATIONS AND APPLICATIONS OF THE EGF/UROTHELIAL EGF-R INTERACTION

Clinical detection and management of TCC, although improving for all stages and grades, has not markedly increased survival rates from this disease. Investigating the interactions between GFs and their urothelial receptors may offer valuable direction for the clinical management of bladder cancer. From the work outlined above, diagnostic, staging and therapeutic approaches can be developed to exploit or to interfere with interactions between EGF and its receptor on TCC.

Diagnostic and prognostic applications of detecting abnormal EGF excretion or EGF-R expression are already being pursued. Because altered EGF-R expression occurs in both low and high grade TCC, as well as on urothelial tissues containing no histologic evidence of malignancy [22,25], it is likely that this "malignant" pattern of receptor expression will precede the development of overt urothelial cancer. If this can be demonstrated, the malignant EGF-R distribution may serve as an intermediate biomarker of risk or exposure. Lack of abnormal EGF-R expression in non-neoplastic hyperproliferative lesions [22], and its presence in unusual bladder neoplasms such as inverted papillomas (which have both the propensity for recurrence and may occur with TCC) [35] would indicate that such applications are promising. Whether abnormal receptor expression is reversible, either spontaneously or through therapeutic/preventative manipulations, is not known. However, the finding that voided urinary EGF levels return to normal following TURBT [18] indicates that this may occur at times. Similarly, while a single measurement of EGF in urine cannot determine if urothelial cancer is present [7,17–19], following urinary concentrations of EGF over time may predict tumor persistence or recurrence [18]. Furthermore, if abnormal EGF-R expression is a reversible phenomenon, then serial measurements of urinary EGF may provide a non-invasive means of monitoring the efficacy of treatments. Finally, identifying greater amounts of EGF-Rs on

tumors may supplement standard histology in predicting future tumor progression [22,23].

Several potentially useful strategies are being developed to disrupt the EGF/EGF-R interaction. Toxins or radioisotopes conjugated to ligands or anti-receptor antibodies, and unconjugated anti-receptor and anti-EGF antibodies, have been used in experimental and therapeutic models of bladder cancer [10,15,20,22,26]. Similar strategies, particularly with isotope conjugates, may also have a role in TCC staging [36]. One example of this approach is already underway after promising *in vitro* work [37–39]. TP-40, the product of a genetically engineered fusion of human TGFα and *Pseudomonas* exotoxin, is being administered intravesically in a multicenter pilot study of patients with superficial bladder tumors, in order to take advantage of the abnormal EGF-R expression associated with TCC.

An alternate therapeutic approach, also based on the observation that the density and distribution of EGF-Rs becomes altered in urothelial cancer, is directed at modifying the urinary/urothelial environment in a way that interferes with EGF binding. Urinary pH varies within a considerable physiologic range (4.8–8.0), and the lower pHs inhibit binding of EGF by its urothelial receptor [3–5,22]. Fukushima *et al.* [40] have reported that urinary acidification alone will prevent promotion and subsequent growth of chemically initiated TCC in rats. Significantly, a variety of proven clinical modalities have been successfully used to modify urinary pH [41]. With this background, the effects of pH upon binding of and response to EGF by human TCC cells have been studied. Messing [22] demonstrated that by reducing the pH of culture media from 7.5 to 5, the total number of EGF-Rs did not change although ligand affinity was decreased by 95%. Similarly, significant growth stimulation by EGF at pH 7.5 was abrogated at pH <7.0, although growth rates in the absence of EGF remained unchanged at lower pHs. Therefore, urinary acidification may be useful for the management and prevention of recurrent bladder cancer.

Additional therapeutic approaches that hold promise include using substances that affect EGF-R expression [39] or that specifically interfere with EGF-R tyrosine kinase activity [42], the actions of subsequent signalling molecules [43], or other steps in receptor functioning [44,45].

Laboratory studies elucidating primary mechanisms for the differential (mitogenic) sensitivity to EGF between malignant and normal urothelium would greatly aid and direct such therapeutic endeavors. Whether such approaches can take advantage of special aspects of urinary tract physiology and anatomy to avoid systemic toxicity for such widespread molecular targets as EGF, its receptor, and signal transduction molecules also must be addressed. However, evidence is building that urothelial carcinoma has evolved by taking advantage of its unique environment; the interaction of uroepithelial cells with urinary EGF is but one example. Advances in treatment and prevention may be most successful if they similarly recognize (and use) these special characteristics.

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